Stress tolerance and hardiness of woody ornamentals

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Ph.D. thesis

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The pictures on the front are from the field experiment with *Hydrangea macrophylla* and *Hydrangea paniculata* (experiment III). Plants were photographed in May at the end of the experiment. On the picture to the right it is clear that most buds of *H. macrophylla*, except those positioned close to the soil surface, had been killed during winter, whereas buds of *H. paniculata* had survived.

Preface

The present thesis has been submitted to the University of Copenhagen in partial fulfilment of the requirement to obtain the Ph.D. degree. The Ph.D. project has been carried out in the time period March 2005 – March 2008 at the Department of Horticulture, University of Aarhus under supervision of Karen Koefoed Petersen, and at the Department of Agricultural Sciences, University of Copenhagen under supervision of Christian R. Jensen and Fulai Liu. In 2007 I additionally spent 5 months at the Department of Horticulture, Iowa State University, USA under supervision of Rajeev Arora.

The thesis deals with stress tolerance and cold hardiness of three woody ornamentals. It is based on a general review on cold hardiness of temperate woody perennials and how water deficit may be used as a mean to increase plant cold hardiness and three manuscripts presenting the main part of the research. The manuscripts will be referred to in the text with their roman numerals (I, II, III).

I owe a debt of gratitude to many people who have helped and encouraged me during my Ph.D. study. First and foremost I would like to thank my supervisors Karen K. Petersen, Christian R. Jensen and Fulai Liu for their friendly guidance, help and constructive criticisms. I have benefited from your individual expertise in different ways, but a common 'theme' has been your interest in my project and willingness always to make time for me, which has been very valuable.

I am also grateful to my supervisor Rajeev Arora for patiently introducing me to protein extraction and SDS-PAGE and for offering to share his extensive knowledge about plant cold hardiness with me. I also appreciate the hospitality of Rajeev and several other people at the Department of Horticulture, ISU during my stay in Ames.

Department of Horticulture, University of Aarhus is a nice place to work with helpful and fine colleagues. I am particularly grateful to Martin Jensen for valuable discussions, for reviewing the *Fuchsia* manuscript and for coming up with the *Hydrangea* idea and to Katrine Heinsvig Kjær for her encouragement and friendship. My thanks also go to Annette Steen Brandsholm for introducing me to the freeze test, and to Lene Korsholm Jørgensen, Department of Agricultural Sciences, University of Copenhagen for learning me how to extract soluble carbohydrates and for skilful technical assistance.

I am additionally grateful to Renate Müller, Department of Agricultural Sciences, University of Copenhagen, Franciszek Janowiak, Institute of Plant Physiology, Polish Academy of Sciences and Olavi Junttila, Department of Biology, University of Tromsø for reading this thesis as the chairman and opponents of the assessment committee.

Contents

Summary

Cold hardiness is a key factor in successful performance of perennial plants in temperate climates. Many introduced horticultural crops cultivated in Denmark originate from other climate zones or temperate regions with a somewhat different climate, and are prone to freezing injuries during winter. This may be due to insufficient maximum freezing resistance and/or because they are adapted to different photoperiodic and temperature conditions in the autumn and spring potentially resulting in ill-timed acclimation and de-acclimation. In a number of horticultural crops knowledge about their hardiness and responses to changing temperature and photoperiodic conditions is therefore of both theoretical and commercial importance. This thesis is based on studies on stress tolerance and cold hardiness of three woody ornamentals.

Since water deficit induces several responses similar to responses observed in cold acclimating plants, water deficit has frequently, although with varying success, been used as a mean to increase plant cold hardiness. In the first study the effects of two types of water deficit; continuous and cyclic were investigated in *Fuchsia magellanica* 'Riccartonii', and it was determined whether water deficit is associated with alterations in cold hardiness. *Fuchsia magellanica* is regarded as one of the hardiest outdoor *Fuchsia* L. species and is sold as a garden shrub, but it is rather susceptible to freezing injuries. In response to continuous or cyclic water deficit *F. magellanica* postponed dehydration by modulating leaf area and closing stomata, whereas only continuous water deficit plants tended to develop modest leaf osmotic adjustment. Water deficiency stimulated formation of abscisic acid (ABA) in the roots, which seemed involved in controlling stomatal closure at mild water deficit. Exposure of *F. magellanica* to low temperatures (8 °C day:4 °C night) and a 10-h photoperiod alone or in combination with cyclic or continuous water deficit did not increase hardiness of either stems or leaves, indicating that *F. magellanica* is freezing sensitive and lacks acclamatory responses to inductive conditions used in the first study. The experiment additionally revealed that roots of *F. magellanica* are less hardy than stems, indicating that more frequent winter survival of roots compared to stems is due to the lesser impact of frost action in the soil.

Hydrangea is widely used and commercially important in landscape gardening. Especially the most-cultivated species *H. macrophylla* is popular due to its attractive flower heads. However, *H. macrophylla* is frequently frost injured, which often causes lack of flowering the succeeding year. Freezing tolerance of different *Hydrangea* species vary widely making the genus useful for studies on comparative frost-hardiness physiology. In the second and third study responses to low, non-freezing temperatures and a reduced photoperiod and cold acclimation and de-acclimation under natural conditions were studied in *H. macrophylla ssp. macrophylla* (Thunb.) Ser. 'Blaumeise' and *H. paniculata* Sieb. 'Kyushu'.

The second study showed that leaves of *H. macrophylla* are more sensitive to chilling temperatures (4 °C) and short photoperiod (10-h) than leaves of *H. paniculata*, as indicated by accelerated senescence and greater perturbations of photosystem II. Short day and low temperature stress was additionally associated with growth cessation, a transient increase in xylem sap ABA concentrations and accumulation of soluble sugars in leaves of both species. *Hydrangea paniculata* furthermore accumulated sugars in stems. Transient increases in xylem sap ABA concentrations were possibly

induced by low soil temperature, with *H. paniculata* responding faster than *H. macrophylla*. Stem cold hardiness of non-acclimated plants did not differ between the two species rendering them equally sensitive to frost episodes in the growing season. As neither species developed increased stem hardiness in response to 25 days of short day and low temperature treatment, it was suggested that cold acclimation of *Hydrangea* stems requires temperatures below ca. 4 °C.

The third study was a field study, which showed that acclimation and de-acclimation of stems of *H. macrophylla* and *H. paniculata* occur approximately simultaneous, but the two species differ significantly in levels of mid-winter cold hardiness and rates of acclimation and de-acclimation. Frequent frost injuries in stems of *H. macrophylla* compared with *H. paniculata* is therefore likely due to lower hardiness mid-winter and during de-acclimation rather than to large differences in timing of acclimation and de-acclimation. In both species increased stem cold hardiness was observed several weeks after growth cessation was detected; suggesting that cold acclimation of *Hydrangea* stems is mainly regulated by temperature. Since increased stem freezing tolerance was not observed until after a time period with several episodes of temperatures below 5 °C and two episodes of sub-zero temperatures, the suggestion from study two, that cold acclimation of *Hydrangea* stems requires temperatures below ca. 4 °C, was supported. In both *H. macrophylla* and *H. paniculata* cold acclimation was associated with starch hydrolysis, accumulation of soluble carbohydrates and altered protein patterns, but the two species displayed clear differences in protein profiles and seasonal accumulation patterns and concentrations of specific soluble carbohydrates. Correlative relationships between increases in xylem sap ABA concentrations, stem freezing tolerance and expression of certain proteins, suggests that ABA is involved in cold acclimation of *H. paniculata* stems. Contrary, ABA did not seem to be involved in cold acclimation of *H. macrophylla* stems.

In conclusion, the first study indicate that it is unlikely, that successful over wintering of aerial parts of *F. magellanica* outdoors in Denmark can be obtained as a result of drought treatments prior to cold stress. Hence, more attention should be paid to variety testing and possibly breeding. The remaining studies contribute to a general knowledge about cold hardiness and stress responses associated with short day and chilling temperatures in *Hydrangea*, which should be taken into account in future work focusing on cold hardiness physiology of *Hydrangea* and the cultivation potential of different *Hydrangea* species.

Sammendrag

Hårdførhed er af afgørende betydning for flerårige planters succes i tempererede klimaer. I Danmark dyrkes mange indførte havebrugsafgrøder, som stammer fra andre klimazoner eller tempererede regioner med et noget anderledes klima, og som er følsomme overfor frostskader. Det kan enten skyldes utilstrækkelig maksimal hårdførhed og/eller, at planterne er tilpasset andre temperaturforhold efterår og forår, hvilket kan resultere i utidige udvikling og ophævelse af hårdførhed. I en række havebrugsafgrøder er viden om planternes hårdførhed og tilpasninger til ændret temperatur og fotoperiodiske forhold således af både teoretisk og økonomisk betydning. Denne afhandling er baseret på studier omhandlende stresstolerance og hårdførhed i tre træagtige prydplanter.

Da tørkestress inducerer adskillige tilpasninger magen til de tilpasninger, som ses i planter, der udvikler hårdførhed, er tørkestress jævnligt, om end med varierende succes, blevet brugt som en metode til at øge planters hårdførhed. I det første studie blev effekterne af to typer af tørkestress; konstant og cyklisk undersøgt i *Fuchsia magellanica* 'Riccartonii', og det blev bestemt om tørkestress er associeret med ændringer i hårdførhed. *Fuchsia magellanica* regnes for værende den mest hårdføre *Fuchsia* L. art og sælges som en prydbusk til haven, men den er temmelig følsom overfor frost. Som respons på konstant eller cyklisk tørke forsinkede *F. magellanica* dehydrering ved at reducere bladarealet og lukke stomata, hvorimod kun planter udsat for konstant tørke udviklede beskeden osmoregulering. Tørkestress stimulerede dannelsen af abscisinsyre (ABA) i rødderne, hvilket, under mild tørkestress, så ud til at være involveret i lukning af stomata. Hverken lave temperaturer (8 °C dag:4 °C nat) og kort daglængde (10 timer) alene eller i kombination med konstant eller cyklisk tørke øgede hårdførheden af *F. magellanica's* stængler eller blade, hvilket tyder på, at *F. magellanica* er frostfølsom og mangler tilpasninger til at udvikle hårdførhed under forhold benyttet i det første forsøg. Forsøget viste desuden, at rødder af *F. magellanica* er mindre hårdføre end stængler, hvilket indikerer, at grunden til at rødder oftere end stængler overlever vinteren skyldes jordens isolerende effekt.

Hydrangea er meget anvendt i privathaver og anlæg og er en kommercielt vigtig prydplante. Især arten *H. macrophylla* er populær på grund af dens store blomsterstande. *Hydrangea macrophylla* får dog jævnligt frostskader, hvilket ofte medfører manglende blomstring det efterfølgende år. Hårdførheden af forskellige *Hydrangea* arter varierer betydeligt og slægten er derfor velegnet til sammenlignende studier af hårdførhed. I det andet og tredje studie blev *H. macrophylla ssp. macrophylla* (Thunb.) Ser. 'Blaumeises' og *H. paniculata* Sieb. 'Kyushus' tilpasninger til lave temperaturer og en reduceret fotoperiode samt udvikling og ophævelse af hårdførhed under naturlige forhold sammenlignet.

Det andet studie viste, at blade af *H. macrophylla* er mere følsomme overfor lave temperaturer (4 °C) og kort daglængde (10 timer) end blade af *H. paniculata*, som antydet af hurtigere ældning og en større reduktion i effektiviteten af fotosystem II. Kort dag og lave temperaturer medførte desuden vækststandsning, en forbigående stigning i koncentrationen af ABA i xylemsaften og akkumulering af opløselige sukkerstoffer i blade af begge arter. *Hydrangea paniculata* akkumulerede desuden sukker i stænglerne. Den forbigående stigning i koncentrationen af ABA i

planternes xylemsaft blev observeret tidligere i *H. paniculata* end i *H. macrophylla* og var i begge arter muligvis induceret af lave jordtemperaturer. Hårdførheden af stængler i ikke-hårdføre planter var ens i de to arter, og de er derfor lige følsomme overfor frostepisoder i vækstsæsonen. Eftersom ingen af arterne udviklede øget hårdførhed som respons på 25 dages kort dag og lav temperatur behandling, blev det foreslået, at udvikling af hårdførhed i *Hydrangea* kræver temperaturer under ca. 4 °C.

Det tredje studie var et markforsøg, som viste, at udvikling og ophævelse af hårdførhed i stængler af *H. macrophylla* og *H. paniculata* sker nogenlunde samtidigt, men at de to arter udviser store forskelle i hårdførhed midtvinter og i hastighederne hvormed hårdførhed udvikles og ophæves. Hyppige frostskader i stængler af *H. macrophylla* sammenlignet med *H. paniculata* skyldes derfor snarere mindre hårdførhed midtvinter og under ophævelse af hårdførhed end store forskelle i timingen af udvikling og ophævelse af hårdførhed. I begge arter sås en signifikant stigning i stænglernes hårdførhed først flere uger efter, at planterne havde indstillet deres strækningsvækst, hvilket tyder på, at udvikling af hårdførhed i *Hydrangea* stængler primært er kontrolleret af temperaturen. Øget hårdførhed af stængler var desuden først observeret efter en periode med adskillige dage hvor temperaturen nåede under 5 °C og to episoder, hvor temperaturen faldt til under frysepunktet, hvilket støtter forslaget om, at udvikling af hårdførhed i *Hydrangea* stængler kræver temperaturer under ca. 4 °C. I både *H. macrophylla* og *H. paniculata* var udvikling af hårdførhed associeret med stivelseshydrolyse, akkumulering af opløselige kulhydrater og ændret proteinmønstre, men de to arter udviste tydelige forskelle i proteinprofiler og sæsonmæssige ændringer i akkumuleringen og koncentrationen af specifikke opløselige kulhydrater. Korrelative sammenhænge mellem stigninger i koncentrationen af ABA i xylemsaft, hårdførhed og udtrykning af visse proteiner tyder på, at ABA er involveret i udvikling af hårdførhed i stængler af *H. paniculata*. Modsat synes ABA ikke at været involveret i udvikling af hårdførhed i stængler af *H. macrophylla*.

Det første studie indikerer, at det er usandsynligt, at succesfuld overvintring af stængler af *F. magellanica* udendørs i Danmark kan opnås ved at tørkestresse planterne før de udsættes for kuldestress. Der bør derfor fokuseres mere på sortsafprøvning og eventuelt forædling. De resterende forsøg bidrager til en generel viden om hårdførhed og stresstilpasninger associeret med kort dag og lave temperaturer i *Hydrangea*, hvilket bør medtages i fremtidige undersøgelser med fokus på hårdførhedsfysiologi og dyrkningspotentialet af forskellige *Hydrangea* arter.

1. General introduction

Rationale

Due to their sessile nature, plants cannot escape adverse environmental conditions and hence diverse mechanisms have evolved to allow their survival. Perennial plants growing in temperate zones are exposed to multiple stresses during winter, low temperature being the most prominent factor affecting their distribution and growth. In temperate climates cold hardiness therefore also to a large extent determines the geographical distribution and cultivation of horticultural crops.

Denmark has a mild temperate climate. The mildness of the climate is largely conditioned by the generally westerly winds and by the fact that the country is virtually encircled by water. Hence, the mean temperature during the coolest months (January and February) is around 0 °C with little fluctuation between day and night temperatures. Sudden changes in wind direction, however, cause considerably day-to-day temperature variations (Danish Meteorological Institute, www.dmi.dk). Due to the mild winters, it is in Denmark possible to cultivate horticultural crops, which are only moderately freezing resistant (i.e. can withstand -15 – (-20) °C) and in many winters even rather freezing sensitive plants, which can withstand down to -5 – (-10 °C), will perform successfully, particularly in protected habitats. Of introduced horticultural crops especially plants originating from areas with a mild unstable climate similar to the Danish, i.e. Central and South Japan and the central area of South China, thrive here. Conversely, woody plants originating from areas with a more stable temperature regime and long-lasting periods of sub-zero temperatures often burst too early in spring, resulting in increased risk of subsequent frost injuries of growing tissues (Ødum 1991). Hence, for some cold hardy woody plants the temperature instability of the Danish climate may be more challenging than the absolute minimum temperatures. For other woody plants, for instance more 'exotic' and less freezing resistant plants, the situation may be the opposite or they may be prone to both freezing injuries caused by minimum temperatures during mid-winter and relatively large temperature fluctuations in the autumn and spring. If autumnal development of hardiness is initiated late, there is an increased risk that an adequate level of hardiness does not develop before the occurrence of the first frost episodes. In numerous horticultural crops knowledge about their hardiness and the dynamics of cold acclimation and de-acclimation and the underlying physiological responses is therefore of both theoretical and commercial importance.

In a number of horticultural crops cultivated in Denmark, freezing injuries caused by insufficient mid-winter cold hardiness, late acclimation or premature de-acclimation have traditionally been mitigated by careful planting in shielded habitats, by covering during winter or in nurseries by keeping the plants in greenhouses or cold stores during winter. However, to avoid both indoor storage during winter and economic losses due to freeze-induced injuries and possibly, though much less manageable, to enhance the marketing potential of relatively freezing sensitive woody plants as garden shrubs it is desirable to be able to manipulate freezing resistance and/or timing of acclimation in some cultures. Factors that increase tolerance against water deficit have frequently been used as a mean to increase hardiness. Hence, water deficit stress or application of abscisic acid (ABA) has resulted in increased hardiness in some woody plants (Chen et al. 1975; Anisko and Lindstrom 1996b; Rinne et al. 1998; Li et al. 2002).

There is a wealth of history in the research area of cold hardiness of plants, and due to the enormous agricultural impact of freezing injury, especially mechanisms associated with development of freezing resistance in herbaceous plants, particularly the model plant *Arabidopsis thaliana*, have been extensively studied. Mechanisms of cold hardiness in woody plants are less known. Additionally, most of the work on woody plants has been conducted on very hardy deciduous trees and coniferous evergreens. Information's on shrubs and moderately or rather freezing sensitive woody plants are fewer.

1.2 Objectives

The overall aim of the studies comprised in this dissertation was to investigate cold hardiness and stress tolerance in some important woody ornamentals. In the first study *Fuchsia magellanica* 'Riccartonii' was used as a model plant. Subsequently two different species of *Hydrangea*; *H. macrophylla* 'Blaumeise' and *H. paniculata* 'Kyushu', differing in hardiness were used. *Fuchsia magellanica* 'Riccartonii' and *H. macrophylla* 'Blaumeise' are both prone to freezing injuries as further outlined in section 3.1. More specifically, the following aims were included:

- To determine effects of different applications of water deficit on growth and selected physiological traits in *F. magellanica* 'Riccartonii'. Secondly, to investigate cold hardiness in *F. magellanica* 'Riccartonii', including determine if water deficit stress is associated with alterations in cold hardiness (I)

- To determine cold hardiness of *H. macrophylla* 'Blaumeise' and *H. paniculata* 'Kyushu' in the non-acclimated state. And to identify physiological responses of the two species grown in controlled conditions to a period of short days and low, non-freezing temperatures to determine whether differences in adaptive responses to short day and low temperature conditions may be related to differences in capacity to cold acclimate (II)

- To study seasonal changes in cold hardiness and selected physiological parameters in *H. macrophylla* 'Blaumeise' and *H. paniculata* 'Kyushu' grown under natural conditions to more accurately establish relations between responses to cold acclimating conditions and cold hardiness (III)

- To determine potential differences in cold hardiness during autumn acclimation and spring de-acclimation in *H. macrophylla* 'Blaumeise' and *H. paniculata* 'Kyushu', to investigate whether greater frost susceptibility of stems of *H. macrophylla* compared to stems of *H. paniculata* is mainly due to differences in mid-winter hardiness, or whether it may also be ascribed to later acclimation and/or earlier de-acclimation (III)

2. General literature review

2.1 Mechanisms of frost survival

Several interchangeable terms are used in the literature concerning frost survival of plants. Here the terms used by Sakai and Larcher (1987) are adopted. Sakai and Larcher defined frost resistance as the ability of a plant to survive subfreezing temperatures. Frost resistant plants employ two major strategies to withstand subfreezing temperatures; freezing avoidance (supercooling) and freezing tolerance. Tissues relying on freezing avoidance display supercooling in which some of the intracellular liquid remains fluid below the freezing point, whereas freezing tolerant tissues tolerate extracellular freezing but avoid intracellular freezing. Often the terms 'frost hardiness' or 'cold hardiness' are used to denote freezing resistance or freezing tolerance. Here the term 'hardiness' is identical to freezing resistance. The introduction is mainly confined to broadleaved trees and shrubs, although reference will also be made to coniferous trees and herbaceous plants where applicable

2.1.1 Freezing avoidance

Supercooled tissues escape frost injury by lowering the freezing point within the cells with antifreeze substances like sugars, alcohols, amino acids and certain proteins or due to the ability to retain water in a liquid phase at low subzero temperatures (deep supercooling). Accumulation of antifreeze substances only lowers the freezing point few degrees, and can be regarded as a shallow supercooling strategy that will not succeed in environments where prolonged subfreezing temperatures exist. In contrast, in plants exhibiting deep supercooling some of the cellular water can supercool to several degrees below 0 ºC by avoiding ice nucleation. The protection is limited to the approximate point of homogenous ice nucleation, which is about -40 ºC. When the capacity for supercooling is exceeded, spontaneous and lethal intracellular freezing occurs (McKersie and Leshem 1994; Guy 2003; Wisniewski 2003).

2.1.2 Freezing tolerance

Freezing tolerant tissues survive freezing by letting ice form extracellularly, which is associated with cellular dehydration. Under most circumstances freezing temperatures induce formation of ice in the extracellular spaces because the intercellular fluid typically has a higher freezing point than the cellular water and usually contains heterogeneous ice nucleating agents (McKersie and Leshem 1994; Guy 2003; Wisniewski 2003). As the vapor pressure of ice is less than the vapor pressure of liquid water, extracellular ice-formation causes a water potential gradient to be established with liquid water moving down the gradient. Consequently, cellular water moves from inside the cell to the extracellular spaces where it freeze, resulting in cellular dehydration. The amount of water lost is dependent on the vapor pressure and hence temperature of the ice (Guy 1990). If the freezing temperature is -10 ºC, the unfrozen liquid will have an osmolarity of about 5 and typically greater than 90% of the osmotically active water will have moved out of the cell (Thomashow 2001). It is clear then, that freezing tolerance must include tolerance to severe dehydration stress.

The distinction between freeze-avoidance and freeze-tolerance strategies is complicated by the fact that many woody plants display freezing avoidance in some tissues and organs, while other tissues and organs undergo extracellular freezing without much supercooling. Typically in woody plants

exhibiting different freezing strategies the bark and vegetative buds will undergo extracellular freezing while xylem cells and flower buds will remain supercooled (Arora et al. 1992; Price et al. 1997; Kasuga et al. 2007).

2.1.3 Categories of freezing resistance

Plants can be differentiated according to their freezing resistance and hardening capacity. Freezing sensitive plants are injured as the consequence of ice formation at rather high sub-zero temperatures (-2 – (-10) °C). Some freezing sensitive plants can enhance their freezing resistance by few degrees, via cellular accumulating of antifreeze substances. Others cannot be frost hardened at all and are injured at very mild freezing temperatures. Plants displaying deep supercooling in some organs or tissues, where the protection is limited to ca. -40 °C, are regarded as partially freezing tolerant even in their frost-hardened state. In fully freezing tolerant plants freezing tolerance is extended to all parts of the plant, and they can, without injuries, survive the lowest temperatures recorded on earth (Sakai and Larcher, 1987). Some boreal woody plants even survive quenching in liquid nitrogen (Strimbeck et al. 2007). Freezing sensitive plants, which are able to increase their freezing resistance, respond directly to decreasing temperatures. Freezing tolerant plants native to the temperate zone show an inherent seasonal hardiness, where development of cold hardiness is correlated with the end of the growing season (Sakai and Larcher, 1987). However, they are also able to acclimate in response to low temperature under long day conditions (Li et al. 2002; Renaut et al. 2005), indicating their ability to cope with sudden temperature drops during the growing season.

Levels of freezing resistance, both in the non-hardened and hardened state, may vary between species, cultivars, ecotypes etc., demonstrating genetic variability for freezing resistance and genetic adaptation to the local climate (Flint 1972; Jensen and Deans 2004; Reyes-Díaz et al. 2005; Vega et al. 2005; III). In addition to genotype- and ecotype-specific differences, levels of cold hardiness may vary between plant organs. In nature shoots are exposed to temperature extremes in the air from which roots are insulated by the soil. Thus, roots are generally less freezing resistant than shoots (Ryyppö et al. 1998; I). Colombo et al. (1995) concluded that in *Picea mariana* seedlings differences in hardiness along the stem and root axes are gradual rather than abruptly differing at the shoot-root interface. A further complication is the difference in cold hardiness exhibited with physiologically ageing. Juvenile plants generally being less frost hardy than mature plants (Arora 2002).

2.2 Regulation of cold acclimation in woody plants

In temperate woody plants cold hardiness is an acquired characteristic by the process of cold acclimation. Cold acclimation can be defined as a process involving physiological and biochemical changes whereby plants become increasingly tolerant to subzero temperatures (Weiser 1970; Li et al. 2004). Parallel to cold acclimation woody plants form buds and develop dormancy (Arora et al. 2003; Rohde and Bhalerao 2007). Dormancy (endodormancy according to the terminology used by Lang 1987) is defined as the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favourable conditions. Growth is possible only after plants have been exposed to a sufficient amount of chilling (Rohde and Bhalerao 2007). The

concurrence of dormancy development, bud formation and cold acclimation often renders it difficult to separate responses responsible for cold acclimation from those associated with dormancy and bud development. In this introduction focus is on cold acclimation and cold hardiness, including both freezing avoidance and freezing tolerance. Dormancy is, however, brought up in few cases where it is found relevant, i.e. in relation to plant water relations and abscisic acid.

2.2.1 Environmental signals

Cold acclimation in freezing tolerant woody plants is generally regarded as a two- or three-stage process. In the first stage a reduced photoperiod triggers growth cessation, formation of terminal buds, development of dormancy and a moderate increase in freezing resistance. In the second stage, freezing resistance is further promoted by low temperatures, especially sub-zero temperatures. Maximum hardiness is obtained most rapidly if the first short day-induced stage of cold acclimation proceeds at rather warm temperatures, as it depends on temperature-dependent metabolic processes. Conversely, the second low temperature-induced stage may be a physical rather than a metabolic process. In some plants a third stage occurs at low freezing temperatures, where hardiness is increased to its maximum (Weiser 1970; Gusta et al. 2005). In many woody plants growth cessation is a prerequisite for the development of cold hardiness (Weiser 1970; Junttila and Kaurin 1990; Olsen et al. 1997). However, cessation of growth is not an absolute requirement for cold acclimation to occur in all plants. In a seasonal study bark and xylem tissues of evergreen peach lacking growth cessation have been shown to develop considerable hardiness (Arora et al. 1992). In leaves, cold acclimation may take place independently of changes in stem elongation growth, as significant levels of hardiness were obtained in deciduous leaves of *Betula pendulata* and *Salix paraplesia* before cessation of elongation growth (Li et al. 2002; 2005a). After cessation of elongation growth, the shoot apex is transformed into a bud.

The effects of reduced photoperiod and low temperature on cold hardiness are often synergistic, their independent effects being limited (Cameron and Dixon 2000; Welling et al. 2002; Li et al. 2005a; 2005b; Mølmann et al. 2005). To reach the maximum level of hardiness a sequential change of shortening photoperiod and low temperature is important, i.e. decrease of photoperiod precedes the lowering of temperature (Weiser 1970; Irving and Lanphear 1967). The synergistic effect of reduced photoperiod and low temperature may be ascribed to induction of different processes (i.e. metabolic and physical as suggested by Weiser 1970). In a study investigating the accumulation of mRNA of a dehydrin gene (*Bplti36*), which was functioning as a marker gene for the cold acclimation process, Puhakainen et al. (2004) additionally suggested that the short day signal sensitize cells to subsequent low temperature exposure. Hence, exposure of silver birch to either short day or low temperature lead to a slight and moderate accumulation, respectively, of *Bplti36* mRNA in leaves, whereas exposure to short day followed by low temperature treatment resulted in a substantial increase in both *Bplti36* transcripts and freezing tolerance, suggesting that short day enhanced the effect of low temperature exposure.

Exceptions to short day control of cold acclimation and dormancy have been reported for some plants. Cold hardiness of stems and buds of raspberry and of rhizomes and buds of cloudberry was only affected by treatment temperature, and no effect of photoperiod could be observed (Kaurin et

al. 1982; Palonen 2006). Likewise, the effect of short day alone on cold acclimation in stems of *Hydrangea macrophylla* and *Hydrangea paniculata* seemed minor (III). In apple and pear development of dormancy was found to be independent of photoperiod. Low temperatures (< 12 °C) induced both growth cessation and dormancy regardless of the photoperiodic regime (Heide and Prestrud 2005). Cold acclimation of roots of woody plants appears to be influenced mainly by the temperature, whereas photoperiod has no or only a minor influence (Ryyppö et al. 1998; Stattin and Lindström 1999). Contrary to observations regarding aerial plant parts, lack of photoperiodic influence on cold acclimation seems to be a general phenomenon in roots.

Phytochrome photoreceptors are involved in detection of changes in photoperiod. Phytocrome exists in two photointerconvertible forms; one absorbs red light (P_r form) and the other absorbs far-red light (P_{fr} form). Phytochrome-regulated responses are therefore initiated by red light and in most cases reversed or blocked by far-red light. P_{fr} is the biologically active form of the pigment, which is destroyed in the dark. The phytochrome determined course of plant growth depends on the duration of the dark period (not the length of the photoperiod) and the red:far-red light ratio (Fosket 1994). In woody plants phytochrome A is believed to be involved in the short day-induced initiation of cold acclimation. Hence, in hybrid aspen overexpression of the oat phytochrome A gene (*PHYA*) rendered the plants insensitive to short photoperiod and prevented growth cessation (under a 6-h photoperiod) and cold acclimation (Olsen et al. 1997). Several lines of evidence have shown a connection between short day-induced growth cessation mediated by phytochrome and the biosynthesis of gibberellins (GAs) and/or sensitivity to GAs. Hence, in woody plants phytochrome may control growth and bud set through regulation of GA biosynthesis. Photoperiodic regulation of GA-biosynthesis was demonstrated in aspen, as stem concentrations of GA_1 decreased in short day treated plants and treatment with the GA-biosynthesis inhibitor paclobutrazol reduced GA-shoot tissue concentrations, which was correlated with complete cessation of growth (Mølmann et al. 2005). Results from experiments with seedlings of *Salix pentandra*, *Betula pendula* and *Populus tremula* x *tremuloides* indicate that it is the conversion of GA_{19} to GA_{20} and further to GA_1 that is under photoperiodic control probably due to transcriptional control of the enzyme GA20-oxidase, which decarboxylate GA_{19} to GA_{20} (Mølmann et al. 2003).

How plants sense the temperature and hence how low temperature-induced cold acclimation is mediated is yet unidentified. Current knowledge indicates that receptor-like protein kinases, two-component histidine kinases, as well as G-protein-associated receptors may represent the potential receptors of cold, drought, salinity or ABA (Xiong and Zhu, 2001).

In addition to photoperiod and temperature the timing and capacity of temperate woody plants to cold acclimate is influenced by a range of factors. Plants may be adapted to their local environment, with timing of cold acclimation changing in a clinal pattern. Hence, in some plants responsiveness to alterations in photoperiod and temperature have been shown to increase gradually with increasing altitude or northern latitude of origin of the plant (Junttila and Kaurin 1990; Li et al. 2002; 2005a). Acclimation and de-acclimation may also be influenced by endogenous annual growth cycles. Compared to acclimation taking place at then end of the growing season cold acclimation may be hindered or the capacity reduced in spring (Fuchigami and Nee 1987; Palonen 2006). Similarly, in *Rhododendron* cold tolerance of leaves declined in spring although plants were retained at low

temperature and short day. However, in addition to the possible influence of endogenous circannual rhythms that may also reflect carbohydrate depletions (Cameron and Dixon 2000). The cold acclimation process may also be modified by nutrient availability (Pellett 1973; Jalkanen et al. 1998), and as further outlined below cold acclimation and hardiness may change according to water availability.

2.2.2 Water deficit

In addition to short day and low temperature especially factors that increase tolerance against water deficit have been shown to increase cold hardiness. Hence, water stress or application of ABA has resulted in increased freezing tolerance in a number of woody plants (Chen et al. 1975; Anisko and Lindstrom 1996a; Rinne et al. 1998; Li et al. 2002; 2003c). However, not al studies have found an effect of water deficit on cold hardiness (van den Driessche 1969; I). The potential magnitude of a water-stress induced increase in cold hardiness may partly depend on a plants maximum attainable hardiness as suggested by Anisko and Lindstrom (1996c). Large increases (8-11 °C) in cold hardiness was observed in stems of red-osier dogwood (Chen et al. 1975; Chen and Li 1978) and *Betula pubescens* (Rinne et al. 1998) after three and 7 weeks of water deficit treatment, respectively. Red-osier dogwood has a maximum freezing tolerance of at least -90 °C (Smithberg and Weiser 1969) and in *Betula pendula* LT_{50} values between -29 – (-38) °C have been observed mid-December (Li et al. 2003b). In *Rhododendron* 'Catawbiense Boursault', which maximum cold hardiness does not exceed ca. -30 °C (Holt and Pellett 1981), 6 weeks of water deficit treatment increased cold hardiness by 0.5-5 °C (Anisko and Lindstrom 1996a; 1996c). And in the most likely freezing sensitive *Fuchsia magellanica* 'Riccartonii' 6 weeks of water deficit treatment followed by four weeks of treatment at short day and low temperature did not affect stem hardiness significantly (I). In addition to the plants maximum level of hardiness, the effect of water deficit on cold hardiness may also be determined by the relative severity of the stress, timing and duration of stress application. Water stress applied for few weeks (Chen et al. 1975; Chen and Li 1978; Anisko and Lindstrom 1996b; Rinne et al. 1998; Li et al. 2002; 2003c) or months (Anisko and Lindstrom 1996c) have been shown to increase cold hardiness of several woody plants, whereas Anisko and Lindstrom (1996c) noticed that continuation of water deficit treatment for more than a year reduced cold hardiness of *Rhododendron* 'Catawbiense Boursault'. In the same study it was additionally observed that the severity (dry *vs.* medium watering regimes) of long-term continuous water deficit treatment influenced cold hardiness differently. The more severe treatment increased hardiness the first year, whereas the second year the situation was reversed. In red-osier dogwood and *Betula pendula* water deficit combined with short day or low temperature had additive effects on freezing tolerance (Chen and Li 1978; Li et al. 2002), whereas in another study concerning red-osier dogwood a combination of water stress and short day did not amplify one another in increasing stem cold hardiness (Chen et al. 1975). The suggestion by Chen and Li (1978) that total cold hardiness results from independent physiological events whose effects are additive therefore seems too simple. The effect of water deficit on cold hardiness may thus depend on a number of factors like severity, duration and timing of application, the plants potential maximum cold hardiness and it may be modulated by environmental signals. The mechanisms whereby water deficit may potentially increase cold hardiness are discussed in section 2.4.

2.3 Physiological components of cold hardiness

2.3.1 Plant water relations

Numerous studies have implied that changes in cold hardiness and plant water relations are related and tissue water content has been observed to decrease in deciduous woody plants during cold acclimation. This dehydration takes place in both stems and buds (Rinne et al. 1998; Li et al. 2003a; III). Tissue dehydration is beneficial in cold acclimating plants as tissues containing a large amount of free water are highly susceptible to formation of lethal intracellular ice (Guy 2003). The water loss observed in cold acclimating plants may be an actual water loss, an apparent loss due to accumulation of carbohydrates or it may result from simultaneous dehydration and increasing dry matter content (Rinne et al. 1998; III). Dehydration and/or alterations of free water to bound water has generally been associated with dormancy development (Wake and Fennell 2000; Arora et al. 2003), and may therefore not be directly related to cold acclimation. However, although it may be a result of dormancy development, dehydration is still an essential component in increased cold hardiness. Welling et al. (2002) suggested that dehydration is a key factor in increased freezing tolerance under short day conditions in stems of aspen. In stems of *Hydrangea macrophylla* and *Hydrangea paniculta*, a probably short day–induced decrease in water content started before any alterations in freezing tolerance was observed, indicating that unaided dehydration is insufficient to increase freezing tolerance in stems of *Hydrangea* (III).

2.3.2 Carbohydrate accumulation

Accumulation of soluble carbohydrates during cold acclimation has been documented in both stems, buds and leaves of broadleaved evergreen and deciduous woody plants (Cox and Stushnoff 2001; Lennartsson and Ögren 2003; Li et al. 2004; Renaut et al. 2004; Eris et al. 2007; III). In turn, in many woody plants cold hardiness has been found to correlate with the total concentration of soluble carbohydrates and/or concentrations of certain soluble carbohydrates (Palonen et al. 2000; Reyes-Díaz et al. 2005; III). In needles of *Picea abies*, *Pinus contorta* and *Pinus sylvestris* Ögren et al. (1997) even found a strong linear correlation between sugar content and cold hardiness across plants within the same genus or family. However, in two species of *Hydrangea*, we found that although both species displayed seasonal changes in concentrations of some soluble carbohydrates which correlated with hardiness, the concentration of soluble carbohydrates was highest in stems of the least cold hardy species (III). Hence, in *Hydrangea* there seems to be no causal relationship between concentrations of soluble carbohydrates and cold hardiness across species. Accumulation of soluble carbohydrates in cold acclimating plants is mainly ascribed to starch hydrolysis, and a close relationship between the degree of starch disappearance and the level of soluble carbohydrates has been observed in some plants (Sauter and van Cleve 1994; Palonen et al. 2000; III). In *Populus tremula* and *Rhododendron* β-amylase genes involved in starch degradation and generation of sugars were shown to be up-regulated in the cambium of dormant trees and in cold acclimating perennial leaves, respectively (Schrader et al. 2004; Wei et al. 2005). Starch reserves are the result of storage of photosynthates during favourable periods like summer and early autumn (Sauter and Kloth 1987; Sauter and van Cleve 1994). In broadleaved evergreen plants and deciduous plants shedding their leaves late accumulation of soluble carbohydrates may also somewhat be directly due to accumulation of photosynthates (Palonen et al. 2000; Lennartsson and Ögren 2002).

In relation to cold acclimation carbohydrates are believed to function as cryoprotectants (Xin and Browse 2000). I.e. the protective function of sucrose and raffinose has been ascribed to their ability to stabilize membranes and proteins in case of freeze-induced dehydration (Crowe 1998; Minorsky 2003). Accumulation of soluble carbohydrates may additionally lower the freezing point of the cell sap due to a lowering of the osmotic potential, thereby reducing the probability of ice crystal formation in the cell (Li et al. 2004). According to Gusta et al. (2004) the rate of freezing in leaves of the herbaceous *Brassica napus* was strongly dependent upon the osmotic potential of the leaves and increasing concentration of cell solutes, especially sugars, greatly depressed the nucleation temperature and slowed down the rate of ice propagation. In xylem parenchyma cells of Japanese white birch, Kasuga et al. (2007) demonstrated that intracellular accumulation of sucrose and its galactosides was associated with an increase in supercooling capability (freezing avoidance). They additionally provided evidence that it is the same types of soluble carbohydrates which accumulate in xylem parenchyma cells and in cortical parenchyma cells during winter. Since cortical parenchyma cells adapt to freezing stress by extracellular freezing, the result suggest that there is no specific difference in accumulation of soluble carbohydrates in tissues displaying different freezing resistance strategies.

Not all soluble carbohydrates accumulate during hardening and soluble carbohydrates that do accumulate may exhibit different patterns of variation during cold acclimation. In woody plants especially sucrose and its galactosides raffinose and stacyose seems implicated in cold hardiness, as their concentrations typically increase and decrease in acclimating and de-acclimating plants (Cox and Stushnoff 2001; Lennartsson and Ögren 2003; III). The involvement of glucose and sucrose in hardiness is less unambiguous. Some studies have found a relation between hardiness and concentrations of glucose and sucrose (Palonen et al. 2000; III), whereas others have found no implication of hexose in cold acclimation (Sauter and Kloth 1987; Imanishi et al. 1998; Cox and Stushnoff 2001). In Japanese white birch Kasuga et al. (2007) noted that sucrose, raffinose and stachyose increased in xylem parenchyma cells during winter. Fructose and glucose also increased but only in the apoplast of xylem tissues. In a short term study maltose was positively correlated with hardiness in *Salix* (Lennartsson and Ögren 2003), whereas in a seasonal study of poplar maltose was shown to be an intermediary compound, increasing from late summer to late autumn, where after it was replaced by more persistent sugars (Sauter and van Cleve 1994). Soluble carbohydrates alone cannot explain cold hardiness. In *Salix* Lennartsson and Ögren (2002) observed that only sugar accumulation following growth cessation is associated with hardening, while accumulation of soluble sugars during cold spells in the growing season does not increase hardiness. And in *Hydrangea paniculata* a shift from 20 °C to 4 °C induced both growth cessation and accumulation of sugars in stems but was not associated with increased hardiness, indicating that other factors limited cold hardening (II).

2.3.3 Protein accumulation

In addition to accumulation of soluble carbohydrates cold acclimation is associated with altered patterns of protein expression. Hence, cold acclimation is associated with synthesis of new proteins and/or up- or down-regulation of existing proteins (Arora et al. 1996; Renaut et al. 2006; III). The group of proteins most extensively documented to accumulate in cold acclimating deciduous woody

plants is dehydrins, a subclass of late-embryogenesis-abundant (LEA) proteins. Accumulation of dehydrins have been reported in a variety of tissue types, and in seasonal studies a decline paralleling de-acclimation in spring is observed (Arora et al. 1992; Muthalif and Rowland 1994; Arora et al. 1996; Rinne et al. 1998; 1999; Welling et al. 2002; Renaut et al. 2004; Dhanaraj et al. 2005; Harris et al. 2006). In leaves of poplar the relative expression of a 60 kDa dehydrin correlated closely with leaf freezing tolerance (Renaut et al. 2004), and in leaves of *Rhododendron* the relative level of a dehydrin varied between genotypes according to their freezing tolerance and within a genotype it indicated the level of freezing tolerance of plants differing in chronological and physiological age (Lim et al. 1999). The precise role and function of dehydrins in plant cells remain unclear, but it is commonly assumed that they assist cells in tolerating dehydration possibly due to stabilizing properties and/or chaperone-like properties (Close 1996; Wise and Tunnacliffe 2004). A study conducted by Rinne et al. (1999) indicated that in apices of cold acclimating birch dehydrins create local pools of water around enzymes in otherwise dehydrated cells, thereby ensuring necessary enzyme activity. However, dehydrins also accumulate in supercooling xylem tissue of *Cornus*, and hence they may not only be related to dehydration caused by extracellular ice formation. Alternatively, supercooling is not a strictly non-dessicative freezing strategy (Karlson et al. 2004). Accumulation of dehydrins has been suggested to be independently regulated by short day and low temperature. Slow accumulation during short day suggests that this regulation is resulting from slow dehydration of the cytoplasm, whereas low temperature induces strong and rapid accumulation of dehydrins (Artlip et al. 1997; Welling et al. 2002). In accordance with the above suggestion, Karlson et al. (2003) observed that in wood of red-osier dogwood accumulation of a 24-kD dehydrin was preceded by short-day induced stem dehydration. Contrary, frost exposure had seemingly no influence on protein accumulation. Recent studies have elucidated different types of dehydrins that are differently regulated by photoperiod and/or temperature. In buds of pubescent birch, the level of *BpuDhn1* increased in response to short day and was further enhanced by subsequent low temperature, whereas *BpuDhn2* was rapidly induced in response to low temperature or water deficit stress when plants were growing or in the ecodormant stage (Welling et al. 2004). Different regulation of different dehydrins may be explained by differences in the promoter region of the underlying genes. Hence, the promoter of a low-temperature induced dehydrin gene in peach contained two DRE/C-repeats, which are responsive to low temperature. In contrast, another dehydrin gene, which was strongly induced by water deficit, contained no DRE/C-repeats (Wisniewski et al. 2006).

Another group of proteins which show distinct seasonal accumulation patterns is bark storage proteins, which act as an overwintering N reserve that is used to support spring growth. During autumnal leaf senescence amino acids derived from leaf proteins are transported to perennating organs like bark and roots where the storage proteins are synthesized. Bark storage proteins therefore accumulate in high amounts during the winter months and are nearly or completely absent during summer (Wetzel et al. 1989; Guak and Fuchigami 2001; Wisniewski et al. 2004). In *Populus* and *Salix* bark storage proteins have been localized to membrane-bound vesicles called protein storage vacuoles (Cooke and Weih 2005). As a result of the close temporal relationship leaf senescence has been suggested to be closely involved in the induction of storage proteins (Sauter and van Cleve 1994). More recent results indicate that expression of bark storage proteins is induced by phytochrome mediated perception of short-day photoperiod, although the involvement

of phytochrome may be indirect. Hence, expression of bark storage proteins may be induced by signals generated due to an altered source-sink relationship resulting from phytochrome mediated growth cessation (Zhu and Coleman 2001; Cooke and Weih 2005). Bark storage proteins definitely play an essential role in seasonal N cycling, which is an important adaptation required for woody plants to enable overwintering. However, whether they play a direct role in cold acclimation, i.e. a regulatory role or a functional role increasing hardiness, is unknown.

In addition to the most-studied dehydrins and bark storage proteins recent studies have shown that other classes of proteins, including heat shock proteins (HSPs), chaperonins and pathogenesis-related (PR) proteins are short day or low temperature inducible in broadleaved woody plants (Lopez-Matas et al. 2004; Renaut et al. 2004; 2006; Wisniewski et al. 2004; Basset et al. 2006). Heat shock proteins and chaperonins have been proposed to be involved in several processes such as refolding of stress-denatured proteins, prevention of aggregation of denatured proteins and membrane protection during thermal fluctuations (Renaut et al. 2006). In chestnut stems a small HSP significantly up-regulated in spring and fall, reaching maximal levels in winter, turned out to be effective in protecting cold-labile enzymes from freeze-induced inactivation (Lopez-Matas et al. 2004). Pathogenesis-related proteins are induced by pathogens and some types of abiotic stress and have traditionally been regarded as having antimicrobial and antifungal roles (Liu and Ekramoddoullah 2006). However, they may display several additional functions. In a study by Ukaji et al. (2004) a so-called WAP18 PR protein isolated from cortical parenchyma cells of mulberry exhibited in vitro cryoprotective activity for a freeze-labile enzyme. The accumulation pattern for WAP18 furthermore showed a strong seasonal tendency, increasing and decreasing in parallel with freezing tolerance. The seemingly wide-spread up-regulation of chaperone-like proteins under short day and/or low temperature conditions indicate that existing and newly synthesized proteins require stabilisation of their structural conformation by molecular chaperones to be translocated correct and/or to fulfil their functions under cold acclimating conditions (Renaut et al. 2006).

2.3.4 Abscisic acid

ABA is thought to be one of the key regulators enhancing cold hardiness. It has been most intensively studied in cold acclimating herbaceous plants, but there is also some evidence for the involvement of ABA in cold acclimating woody plants. The role of ABA in plant cold acclimation was recently reviewed by Gusta et al. (2005). ABA was discovered in search for regulatory signals controlling the onset of dormancy and factors regulating the formation of the abscission zone in fruits and leaves. It turned out that in most plants leaf and fruit abscission layer formation is initiated by ethylene not ABA, but the close involvement of ABA in seed dormancy is now well established (Fosket 1994; Kermode 2005). Traditionally ABA has also been regarded as being implicated in dormancy in woody plants, although its influence is controversial (Le Bris et al. 1999; Rohde and Bhalerao 2007). After its discovery ABA has been shown to be synthesized in response to many kinds of stress. It is particularly known for its central role in root-to-shoot signalling in water deficit plants (Davies et al. 2005; I).

At least four lines of evidence indicate that ABA may play a central role in cold acclimation (Xin and Browse 2000). First, ABA sprayed onto plants can accelerate cold acclimation under natural

cold acclimating conditions and under short day or low temperature conditions (Guak and Fuchigami 2001; Li et al. 2003c) and improve freezing tolerance of different tissues of woody plants grown under a long day regime and at normal growth temperatures (Welling et al. 1997; Rinne et al. 1998; Li et al. 2003c). Second, endogenous ABA levels are elevated under natural cold acclimating conditions and in response to short days and low temperatures (Bertrand et al. 1997; Rinne et al. 1998; Li et al. 2002; 2003b; III). Third, ABA-deficient mutants or plants that have decreased endogenous ABA content due to blockage of ABA synthesis show delayed or reduced development of freezing tolerance (Welling et al. 1997; Rinne et al. 1998) and fourth, many low-temperature responsive genes are induced by ABA (Gusta et al. 2005; Xiao et al. 2006). As a signal transducer ABA has been suggested to be important for controlling the rate and degree of cold acclimation, i.e. earlier cold acclimation and/or greater cold hardiness may be related to faster or more prominent alterations in ABA levels (Li et al. 2005a; 2005b).

In a number of field-grown woody plants, elevated ABA concentrations in xylem sap have been observed during winter, indicating that ABA biosynthesis and/or ABA transport from the root system is stimulated by cold acclimating conditions (Davison and Young 1974; Alvim et al. 1976; Bertrand et al. 1997; 1999; III). In *Hydrangea macrophylla* on the other hand the xylem sap ABA concentration decreased slightly during autumn and winter (III), indicating that cold acclimating conditions is not correlated with increased xylem sap ABA concentrations in all woody plants. Increased ABA xylem sap concentrations have been suggested to be involved in both dormancy (Davison and Young 1974; Alvim et al. 1976) and cold acclimation (Bertrand et al. 1997; 1999). Since xylem sap ABA concentrations of overwintering woody perennials may be elevated for several months, it seems likely that ABA from the root system is not strictly associated with dormancy. Additionally, woody plants are often released from dormancy (endodormancy) earlier than the xylem sap ABA level has been shown to decrease. It is possible, that increased xylem sap ABA concentrations is a direct effect of cold-induced dehydration caused by soil freezing or root chilling, since desiccation of root tips results in accumulation of ABA (Loewenstein and Pallardy 2002; Davies et al. 2005; I). In aerial plant parts ABA has also been shown to increase under conditions leading to increased cold hardiness (Odén and Dunberg 1984; Welling et al. 1997; 2002; Rinne et al. 1998; Li et al. 2002). Results obtained with hybrid aspen overexpressing the oat phytochrome A gene (*PHYA*) indicate, that at least in aerial plant parts the ABA level is controlled by different mechanisms during short day and low temperature induced acclimation (Welling et al. 2002). In buds of short day treated wild type plants the ABA concentration was lower than in long day treated plants, whereas daylength had no clear effect on ABA content in hybrid plants, which are insensitive to photoperiod. Low temperature on the other hand induced a transient increase in ABA content in all plants. Li et al. (2002; 2005a; 2005b) reached a similar conclusion after having observed that in the shoot apex of *Betula pendula* and in leaves of *Salix paraplesia* and sea buckthorn short day and low temperature treatment increased endogenous ABA concentrations, but the accumulation patterns differed depending on the treatment. Low temperature induced a transient increase, whereas short photoperiod induced a gradual increase which levelled of after some days. Combined short day and low temperature treatment induced two transient increases. Contrary to what was observed in buds of aspen (Welling et al. 2002), the ABA level in buds of short day treated *Betula pubescens* increased transiently (Welling et al. 1997; Rinne et al. 1998). Besides cold acclimation ABA in buds has also been related to dormancy. In a study concerning endodormant

buds of *Rosa hybrida* it was concluded that *in situ* biosynthesis of ABA is required for the maintenance of bud dormancy (Le Bris et al. 1999). However, the study also indicated that the buds additionally contained a large pool of ABA likely to have originated from other parts of the plants. Where ABA is produced in cold acclimating plants is unknown, but in addition to endodormant buds ABA biosynthesis can take place in root and leaf cells and external ABA can be taken up by roots. ABA found in the xylem may originate directly from roots or from other tissues, from where it can be loaded into the phloem and transported to the roots (Davies et al. 2005).

Different accumulation patterns of ABA in different tissues and in xylem sap, possibly modulated differently by environmental signals, not only reinforces that the involvement of ABA in cold hardiness is far from straight forward, it also raises questions about ABA biosynthesis, degradation and transport during cold acclimation. Additionally, although it is possible to quantitate the total amount of ABA in tissue or xylem sap, it is not know what the critical concentration is at the active site or what the active site is (Gusta et al. 2005). Besides the absolute concentration it is possible that the action of ABA is influenced by the tissue sensitivity, which may change depending on developmental stage or may be modulated by environmental factors. Hence, in developing seeds both the level of ABA and the sensitivity of the embryo to ABA declines during development (Kermode 2005), and in willow buds the sensitivity to applied ABA have been shown to increase during short day treatment and decrease during subsequent chilling (Barros and Neill 1986).

The involvement of ABA in cold acclimation is related to a controlling role in gene expression and hence the resulting protein levels and patterns (Wisniewski et al. 2006). Analysis of *Arabidopsis* have shown that many ABA inducible genes contain a conserved sequence (*cis*-element) named ABRE (ABA Responsive Element) in their promoter regions (Yamaguchi-Shinozaki and Shinozaki 2006). In *Arabidopsis* Seki et al. (2002) found 22 genes that were induced by both drought, cold and high-salinity. Of those 18 contained the ABRE element and were probably ABA-inducible, and 16 genes contained another element called DRE (Dehydration Responsive Element), indicating that they are part of another response pathway, which is not directly induced by ABA. Likewise in *Populus* there was a significant overlap between ABRE- and DRE-containing gene promoters (Benedict et al. 2006). Hence, cold-inducible genes may contain a number of *cis*-elements in their promoters and their expression therefore mediated by both ABA-dependent and ABA-independent regulatory systems. A similar suggestion was formulated in a study concerning birch, where Rinne et al. (1998) observed that ABA-deficient birch could develop some freezing tolerance under short day without any increase in ABA level.

The DREB/CBF transcription factors are the most-studied transcription factors in relation to cold-induced gene expression. These proteins specifically bind to the above mentioned DRE-element and two other *cis*-elements called the CRT (C-Repeat) and LTRE (Low Temperature Response Element), which have been found in promoters of multiple cold induced genes, and activate the transcription of downstream cold-responsive genes. So far, three cDNAs encoding DREB/CBF proteins have been isolated (DREB1B/CBF1, DREB1C/CBF2 and DREB1A/CBF3), and recently an upstream transcription factor called ICE1 that regulates expression of DREB1A/CBF3 was isolated. ICE1 do not regulate expression of other DREB1/CBF genes, indicating that there are

different expression mechanisms among different DREB/ CBF genes (Yamaguchi-Shinozaki and Shinozaki 2005; Van Buskirk and Thomashow 2006; Yamaguchi-Shinozaki and Shinozaki 2006).

In addition to activation of genes containing the ABRE element, ABA may also induce the expression of DREB/CBF genes but to a significantly lower level than that with cold induction. Similarly ABA also slightly enhance the expression of ICE1, indicating that ICE1 may also regulate ABA–mediated expression of DREB/CBF genes (Chinnusamy et al. 2006 and references therein). The expression of cold-inducible genes is further complicated by interactions among different types of *cis*-acting elements resulting in cross-talk between different signals (Narusaka et al. 2003). The majority of the work concerning gene expression in cold acclimating plants has been conducted using *Arabidopsis* and to a lesser extent other herbaceous plants as models. However, recently the orthologous genes of DREB/CBF has been isolated in sour cherry, *Populus* and in wild and cultivated grape (Owens et al. 2002; Benedict et al. 2006; Xiao et al. 2006). In grape three DREB/CBF genes were isolated and ABA induced transcription of two of them, confirming that some DREB/CBF genes are ABA sensitive (Xiao et al. 2006).

Although there have been no attempts to investigate such interactions in cold acclimating plants, an increasing amount of evidence reveal links between response pathways for ABA, soluble sugars and osmotic stress, suggesting that sugars and ABA may also interact in cold acclimating plants (Gusta et al. 2005). Sugars have been shown to affect ABA metabolism and *vice versa*. I.e. glucose may slow down ABA breakdown, and ABA may stimulate sucrose formation and affect the rate of starch-sugar conversion by altering α-amylase levels (Gibson 2004 and references therein). Sugars and ABA may also influence transcription of the same genes; in *Arabidopsis* seedlings it was shown that transcription of three genes is regulated by multiple signals including glucose and ABA, but probably distinct signalling pathways are used for each signal (Arroyo et al. 2003). According to Rook et al. (2006) synergistic effects of sugar and ABA on gene expression may be due to the involvement of two distinct promoter elements responding to a sugar signal and ABA, respectively, which may induce low gene expression separately, but give a manifold stronger induction when present together.

2.4 Common adaptations between water deficit stress and cold acclimation

As described in section 2.1.2 freezing tolerance is associated with cellular dehydration. So is water deficit stress and osmotic stress is also regarded as one of the negative effects of salt stress on plants. Ion-specific effects being the other (Munns 2002). Hence, a relationship between tolerance to freezing, drought and salinity in plants has been extensively considered in the literature, and a number of studies show the existence of cross-tolerance to osmotic stress in plants; exposure of a plant to moderate osmotic stress may induce resistance to other types of osmotic stresses (Anisko and Lindstrom 1996a; Sabehat et al. 1998; Munshaw 2004). Water deficit induces several responses similar to those changes observed in cold-acclimating plants, and a number of mechanisms have been proposed to explain a water stress-induced increase in cold hardiness. Li and Weiser (1971) suggested that dehydration increases cold hardiness by removal of nonessential water which could participate in formation of damaging ice crystals during freezing. Anisko and Lindstrom (1996a)

hypothesized, that a dehydration induced loss of apoplastic water results in increased cell wall rigidity, which, through the development of a negative turgor pressure at subfreezing temperatures, increases resistance to freeze dehydration via restricted migration of symplastic water. In foliage of red spruce moderate water stress lead to a short term increase in cold hardiness, which correlated with depletion of foliar starch and a slightly increased content of soluble sugars (Amundson et al. 1993). Similarly, in leaves of cabbage seedlings a water deficit induced increase in cold hardiness was accompanied by increasing concentrations of sucrose, glucose and fructose. Hence, Sasaki et al. (1998) suggested that the increased sugar concentrations could contribute to lower the osmotic potential and hence the freezing point of the cell sap, and additionally they could have a protective function by stabilizing proteins and membranes against freezing injury. In oat Maldonado et al. (1997) correlated water deficit induced accumulation of proline and sucrose with increased freezing resistance. However, in cold acclimating *Arabidopsis* Wanner and Junttila (1999) noted that proline accumulation lagged behind enhanced freezing tolerance, suggesting that proline does not play a role in the enhancement of freezing tolerance, although it may be important in longer-term adjustments to low temperature-induced drought stress.

In addition to water deficit-induced responses, which have been directly correlated with increased freezing resistance, water deficit and cold acclimating plants also share a range of other adaptations, including induction of LEA-proteins (Close et al. 1996; Sabehat et al. 1998) and increased endogenous levels of ABA (Li et al. 2002; I). The close association between freezing tolerance and dehydration was noticed by Blödner et al. (2004), who observed that frost tolerant seedlings of *Picea abies* were more drought tolerant than seedlings from a less tolerant progeny, indicating that freezing tolerance and drought tolerance are co-occurring traits. In conclusion, several responses may be involved in a water deficit-induced increase in freezing tolerance, including tissue dehydration, accumulation of soluble carbohydrates and possibly proline and induction of LEA-proteins. Some of these, or other, changes may be mediated by ABA.

As discussed in section 2.2.2 the effect of water deficit stress on freezing resistance may wary depending on several factors related to the treatment and the acclimation potential of the plant. In woody plants water-deficit induced increases in freezing resistance have been constructive, 8-11 °C, in the most hardy plants but slighter in less freezing resistant plants after weeks of treatment. Hence, in practice water deficit stress as a tool to increase freezing resistance may be difficult to manage in an effect full way in terms of timing and severity, and the outcome may be limited. Especially in rather freezing sensitive plants, which usually are the most desirable to be able to 'manipulate' in terms of increased hardiness and/or of timing of acclimation. In the horticultural industry it is a common belief that withholding watering improves “hardening-off”. Although the single effect of withholding water may be limited in terms of improving hardiness, it seems likely though that continuous watering and fertilization in the autumn may cause problems in terms of growth cessation, development of dormancy and cold acclimation, as it may to some extent override the environmental signals. Hence, in some cultures nurseries undoubtly experience a trade-off between having a saleable, good-looking product by the end of the season *vs.* reducing fertilization and watering to allow cold acclimation and hence proper timing may be a challenge.

3. Materials and methods

3.1 Plant material

The plant material chosen for the three experiments included in this thesis was *Fuchsia magellanica* Lam. 'Riccartonii' (I), *Hydrangea macrophylla ssp. macrophylla* (Thunb.) Ser. 'Blaumeise' and *Hydrangea paniculata* Sieb. 'Kyushu' (II; III). *Fuchsia magellanica* 'Riccartonii' and *H. macrophylla* 'Blaumeise' were both chosen because they are prone to freezing injuries even in Denmark's mild climate. The much more cold hardy *H. paniculata* 'Kyushu' was included as a comparison to *H. macrophylla* 'Blaumeise' in order to investigate differential responses to short day and low temperature treatment and to natural cold acclimating conditions. The involvement of *H. paniculata* additionally made it possible to investigate whether frequent frost injuries in *H. macrophylla* compared with *H. paniculata* is mainly due to less mid-winter cold hardiness or whether it can also be ascribed to large differences in timing of acclimation and de-acclimation.

Fuchsia magellanica is a perennial, deciduous *Fuchsia* originating from the Southern Andes in the southern regions of Argentina and Chile (Berry et al. 2004). *Fuchsia magellanica* is regarded as one of the hardiest outdoor *Fuchsia* L. species, but aboveground parts can be frost-injured or die back to the ground in winter and the new growth in spring, even on mature plants, is frost-tender. To our knowledge no studies have been conducted investigating cold hardiness in *Fuchsia*, but according to Danish plant producers *Fuchsia magellanica* only tolerates few degrees of frost without risk of frost injuries and in nurseries it is therefore usually overwintered in cold stores at about -2 °C. On the other hand, *Fuchsia magellanica* is a relatively common garden shrub in Denmark, indicating that a least part of it frequently survives winter. It may however only be belowground parts which survive the winter and is able to produce new shoots in spring. The experimental work with *F. magellanica* was initiated based on a request from Danish plants producers, who wished to be able to overwinter *Fuchsia* outdoor.

The great ornamental value of *Hydrangea* has made this genus famous, widely used and commercially important in landscape gardening. Especially the most-cultivated species *H. macrophylla* ssp. *macrophylla* (Thunb.) Ser. is popular due to its attractive flower heads. Flower buds of most *H. macrophylla* varieties are formed during the autumn and over-winter on dormant stems, flowering will therefore only occur the succeeding year if terminal and/or lateral flower buds are present and undamaged. However, it is a common problem that *H. macrophylla* does not flower during summer and insufficient cold hardiness is believed to be the main reason. According to few scientific studies (Suojala and Linden 1997; Adkins et al. 2003), and the experience of *Hydrangea* producers, *H. paniculata* is considerably more cold hardy than *H. macrophylla* when plants are well-hardened, making the genus useful for studies on comparative frost-hardiness physiology. Although insufficient mid-winter hardiness may account for part of the frost injuries encountered in *H. macrophylla* it is possible that part of the problem can be ascribed to late acclimation in fall and/or premature de-acclimation in spring. Partly because *H. macrophylla* is considered to acclimate late and de-acclimate early (Adkins et al. 2003), and partly because temperatures below -18 °C – (-24 °C), which corresponds to the maximum hardiness of stems of 10 *H. macrophylla* cultivars (Adkins et al. 2003), are rather unusual in Denmark, whereas frost injuried *H. macrophyllas* are rather common. *Hydrangea paniculata* is native to south eastern China and Japan

and *H. macrophylla* is native to Japan. In their indigenous habitats *H. paniculata* has a broader altitudinal distribution than *H. macrophylla*, i.e. *H. paniculata* is found from 500-1000 m in China and from sea level to 1200 m in Japan, while *H. macrophylla* is distributed from sea level to 150 m (McClintock 1957).

3.2 Growth conditions and treatments

Three pot experiments were performed. The first was focusing on drought adaptations and the potential effect of two different types of water deficit stress on cold hardiness in *Fuchsia magellanica* 'Riccartonii'. Drought responses were investigated after 6 weeks of pre-treatment, where individual plants grown in a greenhouse (17-h photoperiod, 21 ºC day and night) under conditions of unrestricted water supply were compared with plants subjected to cyclic or continuous water deficit. After additional 4 weeks of treatment at short day (10-h) and low temperature (8 °C/4 °C day/night), freezing tolerance was examined. Continuous water deficit plants were given an amount of nutrient solution equal to 40% of mean daily water consumption, while cyclic water deficit treatment was a repeating non-lethal cyclic water deficit treatment alternating between stress phases, where watering was withheld, and recovery phases where the plants received the same amount of water and nutrient solution as control plants for five days.

The second experiment, involving *Hydrangea macrophylla* 'Blaumeise' and *Hydrangea paniculata* 'Kyushu', was a time-course climate chamber experiment, focusing on hardiness in the non-acclimated state and adaptive responses of plants subjected to a controlled short day (10-h) and low temperature (4 °C) regime compared to control plants grown at 20 °C and a long day (18-h). Since 25 days of short day and low temperature treatment did not induce increased stem cold hardiness in the two *Hydrangea* species, the third experiment was a field experiment, where cold hardening was clearly induced. In the field experiment plants were kept outdoors at Research Centre Aarslev, Denmark (latitude 55° 01' N, latitude 9 m) and current year shoot tissue was sampled at 7 or 8 times from ultimo September 2006 to the beginning of May 2007.

The physiological components considered and the methods and instruments used during the experiments are shortly summarized below. More details are available in the manuscripts.

3.3 Methods

3.3.1 Freezing tolerance

Freezing tolerance of stems (I, II, III) and roots (I) was measured by exposing plants to controlled freezing after which injuries were estimated by the ion-leakage method. After rinsing in tap water and demineralised water the samples were placed in 70-mL test tubes and incubated in a controlled freezer. A small amount of demineralised water was added to each sample to initiate ice formation. The freezer cooled at a rate of maximum 5 ºC per hour to 0 ºC and subsequently at 2 ºC per hour until the selected temperature was reached. The selected temperature was maintained for 2 h, where after the samples were withdrawn and thawed at 4 ºC. Ions were extracted with 35 mL demineralised water for 20-24 h at room temperature and the electrical conductivity measured (EC_{frozen}) using a ION570 ISE-meter with temperature-corrected display (Radiometer, Copenhagen,

Denmark). After determination of the electrical conductivity the samples were autoclaved for 1 h to allow maximum leakage of ions. After autoclaving the samples were allowed to cool to room temperature and the electrical conductivity was measured again ($EC_{autoclave}$). The electrical conductivity of demineralised water (EC_{water}) was measured to give the zero level of electrical conductivity. Relative electrolyte leakage (REL) was calculated as $REL=(EC_{frozen}-EC_{water}) * 100/(EC_{autoclave}-EC_{water})$. Freezing tolerance was estimated as LT_{50} values, the temperature representing 50% relative electrolyte leakage, as explained in section 3.3.8.

3.3.2 Leaf area and elongation growth

Leaf area (I) was determined using an area meter (LI-3000, Lambda Instruments Corp., Lincoln, NE). Shoot elongation growth (II, III) was determined with a ruler measuring the distance between the shoot tip and a reference mark made between the tip and the first node.

3.3.3 Water status measurements

Leaf water potentials (I, II) were measured with a pressure chamber [SKPM 1400, Skye Instruments Ltd., Llandrindod Wells, UK (I) and Soil Moisture Equipment Corp., Santa Barbara, CA, USA (II)], and leaf osmotic potentials (I) were measured using Psychrometers (C-52 sample chambers, Wescor Inc., Logan, UT). Leaf turgor pressure was calculated as the difference between leaf water potential and leaf osmotic potential. Stomatal conductance (I) was determined using gas exchange (CIRAS-2 infrared gas analyser, PP systems, Hertfordshire, UK). Fresh and dry weights of leaves (I) and stems (III) were recorded before and after drying to constant weight at 80 °C.

3.3.4 Chlorophyll fluorescence and chlorophyll content

Fluorescence emissions (II) were measured using a portable chlorophyll fluorometer (Handy-PEA, Hansatech Instruments, King´s Lynn, Norfolk, UK), and chlorophyll contents (II) were estimated using an optical leaf-clip (ADC:OSI CCM 200 Chlorophyll Content Meter, Hoddesdon, Herts, England).

3.3.5 Carbohydrates and proline

Soluble carbohydrates in leaves (I, II) and stems (II, III) were extracted from powdered, freeze-dried material using 80% (v/v) ethanol and quantitatively determined using high-performance liquid chromatography (HPLC, Hewlett Pacard 1047A, Waldbronn, Germany). Concentrations of starch in leaves (I) and stems (III) were determined in the remaining pellets after extraction of soluble carbohydrates. The starch was hydrolyzed, purified, dried and after re-dissolving the resulting glucose content was determined on HPLC. Proline concentrations in leaves (I) were determined colorimetrically by the acid-ninhydrin procedure.

3.3.6 Xylem sap collection and ABA measurements

Xylem sap from root systems (I, II, III) was collected using pressure chambers. When collecting sap from *Fuchsia magellanica* 'Riccartonii' a smal chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) was used, whereas in experiment II and III a larger self-made (10 l) Scholander-type pressure chamber was used. Xylem sap was collected by pressurising the whole root system at a pressure close to the value of the leaf water potential (I, II). Concentrations of ABA in xylem sap (I, II, III) was measured without purification by an enzyme linked immunosorbent

assay (ELISA) using a monoclonal antibody for ABA. To verify that the xylem sap did not contain compounds which reacted with the antibody other than the antigen a cross reaction test was performed for both *F. magellanica* and the two *Hydrangea* species.

3.3.7 Protein analysis

Bark proteins (III) were extracted from powdered lyophilized bark using phenol-extraction followed by precipitation with ammonium acetate in ethanol. Proteins were also attempted extracted using a "borate buffer" and TCA-precipitation. The protein concentration in the extracts was assayed using the method of Esen (1978), and the protein accumulation patterns were studied in Coomassie-stained one-dimensional SDS-PAGE.

3.3.8 Statistics

Freezing tolerance of stems (I, II, III) and roots (I) was expressed as LT_{50} values assessed as temperatures representing 50% REL. In experiment I LT_{50} values were estimated by fitting data by regression analysis (PROC NLIN of SAS) to the sigmoid function $y=y_0 + a/(1+\exp(-(x-x_0)/b))$, where y is REL with a value of y_0 at the lower asymptote, x is the temperature with a value of x_0 at the inflection point representing 50% injury (LT_{50}) and *a* and *b* are parameters. Differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap. To determine whether LT_{50} estimates differed between stems and roots (I), REL data for both stems and roots were fitted by regression analysis (PROC NLIN) to the sigmoid function $y=y_0 + a/(1+\exp(-(x-(x_0 + x_0\text{rod}*\text{rod})/b))$, where y, y_0, x, *a* and *b* are the same parameters as described above and $x_0$rod is the difference between stem and root LT_{50} estimates. The difference in LT_{50} between the two tissue types was taken as significant if it differed significantly from 0. In experiment II and III REL values were fitted by regression analysis (PROC NLIN of SAS) to the sigmoid function $REL=REL_{min} + (REL_{max}-REL_{min})/(1+\exp(c*(d-T)))$, where REL_{min} is the base line of REL, REL_{max} is the maximum REL, c is the slope of the function at the inflection point d and T is the treatment temperature. The temperature (d) at the inflection point was used as LT_{50}. Like in experiment I differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap. To support the LT_{50} estimates in experiment I stem REL data were subjected to two-way analysis of variance and non-parametric Kruskall-Wallis tests to determine the main effects of treatment and freezing temperature on REL. In experiment II stem REL data were tested using both a four-way analysis of variance and Kruskall-Wallis tests, to determine whether REL data differed between species, treatments, species and time (treatment duration). As no differences between species where detected, REL data were pooled across species for each treatment and time separately before estimating LT_{50} values.

The effects of treatment (I, II), time (II, III) and species (II, III) on a range of parameters were examined using one-way (I), two-way (III) or three-way (II) analysis of variance depending on the number of variables (PROC GLM of SAS). Heterogeneities of variances were tested using Bartlett's test. When necessary, logarithmic transformations were performed to ensure homogeneity of variance. Differences between individual means were identified using Tukey's Studentized Range (I, III) or the Tukey-Kramer test (II) at the 5% significance level. If the assumption concerning homogeneity of variance was not fulfilled data were also tested using a non-parametric Kruskall-Wallis test.

Linear relationships between root water potentials and xylem sap ABA concentrations (I), concentrations of soluble carbohydrates and LT_{50} values (III) and between number of hours with a mean temperature < 5 °C and LT_{50} values (III) were examined using Pearson's correlation coefficient (PROC CORR of SAS). A relation between xylem sap ABA concentrations and stomatal conductances was evaluated by a logarithmic function. The parameters of the curve were derived by the non-linear least-squares iteration procedure (PROC NLIN), and the coefficient of determination (r^2) was calculated as 1-SSE/CSS where SSE is the residual sum of squares and CSS is the corrected total sum of squares.

4. Results and discussion

The three studies included in this thesis are all discussed in detail in the succeeding manuscripts (I, II, III) and the results have been related to the existing literature in the introduction. Therefore, only a few additional results, the main conclusions and perspectives are discussed in this chapter.

4.1 Water deficit stress affects growth and physiology but not freezing tolerance of the most likely freezing sensitive *Fuchsia magellanica* 'Riccartonii' (I)

Six weeks of continuous or cyclic water deficit stress had pronounced effects on growth and physiology of *F. magellanica* 'Riccartonii'. Both continuous and cyclic water deficit plants postponed dehydration by reducing the leaf area and closing stomata (Fig. 2, Table 1, I), which are common observations in water deficit plants (Chaves et al. 2003; Yin et al. 2004). Modulation of the leaf area and stomatal closure was sufficient for continuous water deficit plants to maintain their leaf water status, whereas significant reductions in the leaf water content and leaf- and root water potentials were observed in cyclic water deficit plants (Table 1, Fig. 3, I), indicating that *F. magellanica* is more sensitive to stress phases of cyclic water deficit than to continuous water deficit. Values of leaf water- and leaf osmotic potentials and leaf turgor pressures indicated that continuous water deficit plants, contrary to cyclic water deficit plants, possessed a modest ability to adjust osmotically. The difference between continuous- and cyclic-water deficit plants in ability to adjust osmotically, may be ascribed to different rates of stress development, as osmotic adjustment is usually a slow process (Chaves et al. 2003). ABA was clearly involved in drought-stress responses in *F. magellanica*. Increasing water deficiency, measured as decreasing root water potentials, stimulated ABA formation in the roots, and root-originated ABA seemed to be involved in controlling stomatal conductance during mild water deficits (Fig. 4, Fig. 5, I).

Neither cold acclimating conditions alone nor in combination with water deficit stress increased stem cold hardiness of *F. magellanica* compared with non-hardened plants (Table 3, I), demonstrating that *F. magellanica* lacks cold acclimation ability under inductive conditions used in experiment I. Since stems are the potential aerial overwintering organs in *Fuchsia*, only data concerning stem freezing resistance was published. However, short day and low temperature treatment alone of in combination with water deficit stress did not increase leaf cold hardiness either (Table 1, unpublished data). Alterations in leaf water status (Table 1, Fig. 3, I) and concentrations of soluble sugars and proline (Table 2, I) observed in water deficit plants could therefore not be related to subsequent alterations in cold hardiness. In fact, short day and low temperature treatment alone of in combination with water deficit stress decreased leaf hardiness slightly, possibly due to preliminary leaf senescence. *Fuchsia magellanicas* lack of cold acclimation ability under inductive conditions used in experiment I suggests that it is freezing sensitive and only can increase its hardiness by a maximum of few degrees. If the acclimation potential of *F. magellanica* is less than 2 °C, which was the temperature interval used in the freeze test, it is possible that we did not uncover an increase in cold hardiness. However, an increase in cold hardiness of less than 2 °C has very limited practical implication. Alternatively, *F. magellanica* may require a different photoperiodic and/or temperature regime to increase its hardiness. Irrespective of *F. magellanica*'s potential to increase its freezing resistance, water deficit treatments did not

increase hardiness of either stems or leaves significantly, indicating that continuous and cyclic water deficit stress is not a useful tool to manipulate cold hardiness in *F. magellanica.*

Table 1. Cold hardiness of *Fuchsia magellanica* 'Riccartonii' leaves estimated as LT_{50} values. Leaves of plantlets were tested after 6 weeks of control, continuous water deficit or cyclic water deficit pre-treatments and additionally 4 weeks of treatment under short day (SD, 10-h) and low temperature (LT, 8 °C day/4 °C night). Except control plants which stayed under a long day/warm temperature regime throughout the experiment. LT_{50} [mean ± SE (°C)] are shown for four plants tested at 10 temperatures. Different letters within the column indicate significant differences between treatments. Differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap.

Treatment	LT_{50} [mean ± SE (°C)]
Control	-6.6 ± 0.3^{b}
Control + SD + LT	-4.4 ± 0.3^{a}
Continuous water deficit + SD + LT	-4.5 ± 0.2^{a}
Cyclic water deficit + SD + LT	-4.5 ± 0.2^{a}

Experiment I furthermore showed that roots of *F. magellanica* are less cold hardy than stems (Table 3, I), which is in accordance with results obtained in other plants (Ryyppö et al. 1998; Stattin and Lindström 1999). The frequent ability of *F. magellanica* to produce new shoots in spring after killing of stems may therefore be ascribed to the lower impact of frost action in the soil rather than greater cold hardiness of roots compared to stems.

4.2 Threshold temperature for cold acclimation in *Hydrangea macrophylla* and *Hydrangea paniculata*? (II; III)

Results obtained in experiment II demonstrate that stem cold hardiness in the non-acclimated state does not differ between *H. macrophylla* and *H. paniculata* (Table 2, II), indicating that they are equally susceptible to cold spells in the growing season. Additionally, neither species developed increased stem freezing tolerance during 25 days of low temperature (4 °C day:night) and short photoperiod (10-h) (Table 3, II). It may be argued that the applied treatment was not optimal for inducing cold acclimation, as the initial photoperiodic control of cold acclimation is related to temperature-dependent metabolic processes, and hence is obtained most rapidly at temperatures higher than 4 °C (Weiser 1970). Simultaneous short day and low temperature treatment, as used in experiment II, may therefore delay initial cold hardening processes including growth cessation and formation of terminal buds and prevent development of maximum hardiness. Growth cessation (Fig. 1, II) but no bud formation (visual observation) was observed in *H. macrophylla* and *H. paniculata*. However, the literature is complete with reports where simultaneous short day and low temperature treatment, similar to the one employed in experiment II, or separate low temperature treatment has yielded cold acclimation in woody plants (Cameron and Dixon 2000; Renaut et al. 2004; Arora and Lim 2005; Li et al. 2005b; Palonen 2006). Hence, it does not seem convincing that simultaneous short day and low temperature treatment completely hindered cold acclimation in *H. macrophylla* and *H. paniculata,* suggesting that the applied treatment was insufficient to induce cold acclimation. It was therefore suggested that cold acclimation in stems of *Hydrangea* may require exposure to temperatures below ca. 4 °C.

In the field, hardiness was clearly induced in both *H. macrophylla* and *H. paniculata*, but in line with the results from the climate chamber experiment, the LT_{50} values indicated that cold acclimation of stems may require exposure to temperatures below at least 5 °C. Ultimo October, when the minimum air temperature reached was ca. 5 °C, neither species showed increased stem freezing tolerance (Fig. 2, III), as indicated by LT_{50} values equivalent to LT_{50} values previously determined in non-hardened plants (Table 3, II). However, approximately one month later, during which several incidents of temperatures below 5 °C and two episodes of sub-zero temperatures occurred, both species displayed a significant increase in stem cold hardiness. Since growth had ceased in both *H. macrophylla* and *H. paniculata* several weeks prior to the initial increase in freezing tolerance, implying perception of a short day signal, it is speculated that cold acclimation of *Hydrangea* stems is mainly regulated by temperature rather than by photoperiod. If that is the case, and if the photoperiod regulates dormancy induction, *Hydrangea* may be an interesting experimental system to study responses associated with short day-induced dormancy *vs.* low temperature-induced development of freezing tolerance. And it additionally explains why *H. macrophylla* is considered to acclimate late. An exact threshold temperature for cold acclimation in stems of *H. macrophylla* and *H. paniculata* is impossible to elucidate from the results obtained in the field experiment, they only suggest that it may be below 5 °C. It is possible, that it was the two frost episodes, where the temperature dropped just below 0 °C, which triggered the increase in stem cold hardiness. Adkins et al. suggested that sub-zero temperatures is an important hardiness-promoting factor in *H. macrophylla*, and Weiser (1970) proposed that frost is the triggering stimulus in the second stage of acclimation in woody plants.

4.3 Effects of chilling temperatures and short photoperiod on PSII function, sugar concentrations and xylem sap ABA concentrations of *Hydrangea macrophylla* and *Hydrangea paniculata* (II)

Chilling temperatures (4 °C) and short photoperiod (10-h) resulted in greater perturbations of PSII and a more pronounced chlorophyll loss in leaves of *H. macrophylla* than in leaves of *H. paniculata*, indicating that leaves of *H. macrophylla* are more chilling sensitive than leaves of *H. paniculata* (Fig. 2, Fig. 3, Fig. 4, II). In both species reduced PSII function was probably partly due to hindered electron transport downstream Q_A^-, as indicated by increased fluorescence intensities at the J step of the O-J-I-P transient (Lazar 2006). Greater chilling tolerance of leaves of *H. paniculata* may be advantageous in terms of cold hardiness as even deciduous trees may benefit from maintaining leaves as long as possible during autumn to allow continued photosynthesis and hence building up of carbohydrate reserves (Lennartsson and Ögren 2002).

Hydrangea paniculata accumulated sucrose and hexoses in both leaves and stems in response to short day and low temperature, whereas in *H. macrophylla* sugar accumulation was restricted to leaves (Fig. 5, II). Since growth ceased in both species (Fig. 1, II), but the efficiency of PSII remained relatively high (Fig. 3; II), accumulation of sugars may have resulted from a shift in the relationship between anabolic and catabolic processes (Renaut et al. 2004; Guy et al. 1992). Accumulation of greater amounts of sucrose and hexoses in *H. paniculata* compared with *H. macrophylla* may be related to its better PSII-functioning and lesser chlorophyll loss. Short day and

low temperature treatment additionally resulted in a transient increase in xylem sap ABA concentrations in both species, indicating stimulated ABA formation and/or transport of ABA from the roots, which is in agreement with earlier findings in other woody plants (Bertrand et al. 1997; Wan et al. 2004). Increasing xylem sap ABA concentrations may be a stress-response to low soil temperature (Starr et al. 2004; Wan et al. 2004), and a faster transient ABA increase in *H. paniculata* than in *H. macrophylla* may suggest faster acquisition of tolerance to low temperature.

Some of the adaptive responses observed in *H. macrophylla* and *H. paniculata* in response to short day and low non-freezing temperatures are similar to responses commonly observed in cold acclimating plants, raising the question why no increase in stem cold hardiness was observed (see section 4.2). However, reduced growth and accumulation of sugars are not only associated with cold acclimation, but are also general responses to abiotic stress. Additionally, accumulation of soluble sugars in overwintering organs (stems) only took place in *H. paniculata* and was dominated by hexoses (glucose and fructose), which subsequently were shown not to be correlated with stem cold hardiness in *H. paniculata* (Fig. 6, III). Similarly, increasing endogenous ABA concentrations is a common response to short day and/or low temperature, and may not strictly be associated with cold acclimation (Li et al. 2002; 2005b; Wan et al. 2004).

4.4 Cold acclimation in *Hydrangea macrophylla* and *Hydrangea paniculata* is associated with species-specific alterations in soluble carbohydrates, proteins and ABA (III)

In the field, *H. macrophylla* and *H. paniculata* acclimated approximately simultaneously and they also started to de-acclimate at the same time (Fig. 2, III), and as expected *H. paniculata* developed considerably greater stem cold hardiness than *H. macrophylla*. Hence, greater frost susceptibility of stems of *H. macrophylla* compared with stems of *H. paniculata* seems mainly to be due to lower mid-winter hardiness than to large differences in timing of acclimation and de-acclimation. However, lower absolute cold hardiness during de-acclimation renders stems of *H. macrophylla* more susceptible to spring frost than stems of *H. paniculata*. Except for two and 7 frost-episodes in early November and late January, respectively, the minimum daily air temperatures fluctuated between 0-10 °C from start of the experiment and until the end of January, when maximum hardiness of stems of *H. macrophylla* and possibly also *H. paniculata*, but that was unclear since LT_{50} values could not be precisely determined on a number of dates, was reached, indicating that neither species requires a period of stable, low temperatures to cold acclimate. The robustness of the two species to temperature fluctuations during acclimation may be ascribed to their maritime origin. The field experiment additionally indicated that buds of *H. macrophylla* are less cold hardy than stems, as most buds were killed in January (visual observation) concurrent with the occurrence of the lowest air temperatures, while stems survived. In January the lowest air temperature recoded was -7.3 °C and the LT_{50} value for stems of *H. macrophylla* was ca. -15 °C, indicating that the difference in cold hardiness between stems and buds may be considerable.

Cold acclimation was associated with stem dehydration, altered expression of bark proteins, starch hydrolysis and accumulation of soluble carbohydrates in both *H. macrophylla* and *H. paniculata* (Fig. 3, Fig. 4, Fig. 6, III). In the autumn the stem water content of both species started to decrease before the most likely low temperature-induced increases in stem hardiness were observed,

indicating that decreasing water content may be more associated with short day-induced dormancy than cold acclimation. In accordance with earlier findings in other woody plants (Palonen et al. 2000; Cox and Stushnoff 2001) concentrations of sucrose and raffinose were correlated with stem hardiness in both species (Fig. 6, III). However, *H. macrophylla* additionally accumulated fructose and glucose during winter, which more resembles results obtained with herbaceous plants and deciduous leaves of woody plants (Guy et al. 1992; Gusta et al. 2004; Li et al. 2004; Renaut et al. 2004). Concentrations of soluble carbohydrates were lowest in the most hardy species (*H. paniculata*), suggesting that the concentration of soluble carbohydrates is not a useful 'indicator' of hardiness between *Hydrangea* species. In *H. paniculata* xylem sap ABA concentrations (Fig. 5, III) were elevated throughout a large part of the cold season, which corresponds to previous observations in other woody plants (Davison and Young 1974; Alvim et al. 1976; Bertrand et al. 1997; 1999). In *H. macrophylla* on the other hand, no such seasonal trend in xylem sap ABA concentrations were observed. Hence, if xylem sap ABA is implicated in cold acclimation (Bertrand et al. 1997; 1999), species-specific differences in cold hardiness in *Hydrangea* may be related to different regulation of cold acclimation.

4.5 Conclusions and perspectives

Based on the experimental results, it was concluded that:

- *Fuchsia magellanica* 'Riccartonii' is most likely freezing sensitive, and roots are more sensitive than stems.
- Continuous water deficit and cyclic water deficit are not useful tools to increase cold hardiness of leaves and stems of *F. magellanica* 'Riccartonii' under the conditions used in this study.
- Cold hardening in stems of *Hydrangea macrophylla* 'Blaumeise' and *Hydrangea paniculata* 'kyushu' seems more influenced by temperature than by photoperiod, and possibly there is a quite low threshold temperature (< 4-5 °C) above which no significant hardening occurs.
- *Hydrangea macrophylla* and *H. paniculata* are equally sensitive to frost while they are growing, and cold acclimation and de-acclimation is initiated approximately at the same time in the autumn and spring. Greater frost susceptibility of *H. macrophylla* stems than of *H. paniculata* stems is therefore mainly due to lower mid-winter hardiness and lower hardiness during de-acclimation.
- Starch hydrolysis and accumulation of sucrose and raffinose is implicated in cold acclimation in both *H. macrophylla* and *H. paniculata* and stem cold acclimation is additionally associated with tissue dehydration and qualitative changes in bark proteins of both species.
- Species-specific differences in cold hardiness of *H. macrophylla* and *H. paniculata* may be related to differences in biosynthesis of ABA in the roots and/or transport of ABA from the root system during winter, since autumnal increases in stem cold hardiness were associated with increasing xylem sap ABA concentrations in *H. paniculata* but not in *H. macrophylla*
- Leaves of *H. macrophylla* are more sensitive to chilling temperatures and short photoperiod than leaves of *H. paniculata* as indicated by greater perturbations of PSII and a more pronounced loss of chlorophyll.

- Adaptive responses of *H. macrophylla* and *H. paniculata* to short day and chilling stress include growth cessation, a transient increase in xylem sap ABA concentrations and accumulation of sugars in leaves of *H. macrophylla* and in leaves and stems of *H. paniculata.*

In search for more hardy *Fuchsias* and/or methods to improve hardiness of *Fuchsia*, more extensive variety testing and potentially breeding is a relevant place to continue. Reports from nurseries indicate, that there may be one or two *Fuchsia magellanica* varieties which are more hardy than 'Riccartonii' (*Fuchsia magellanica* 'Georg' and 'Karen'), but their hardiness have never, to my knowledge, been determined. Determination of cold hardiness of the traditional outdoor *Fuchsia magellanica* varieties at a number of occasions during the cold season could rank them according to hardiness and give an indication of their capacity to enhance their freezing resistance. If any of the *Fuchsia magellanica* varieties have capacity to enhance their freezing resistance, the exact inductive conditions are unknown, and hence field trials where plants are subjected to natural cold acclimating conditions seems most suitable. However, field trials include risks of severe frost injuries or killing of plants, and hence it may be necessary to keep the plants indoor part of the time.

The present study has indicated the answer to some questions regarding cold hardiness in *Hydrangea* and uncovered a lot more. Future studies should focus on the following aspects to elucidate the crucial components in *Hydrangea* cold hardiness and to be able to asses the cultivation potential of different species in Denmark and potentially other temperate climates.

- The involvement of photoperiod *vs.* temperature in induction of cold acclimation by investigating their separate effects and sequential effects in factorial experiments performed under controlled conditions.
- The existence of a possible threshold temperature for cold acclimation in *Hydrangea*. I.e. how low temperature is required for *Hydrangea* to cold acclimate and what is the minimum 'exposure' time.
- Cold hardiness of *Hydrangea* buds *vs.* stems to determine whether buds are much less freezing tolerant than stems. It would additionally be interesting to study cold hardiness of different tissues within organs to determine the primary spots of injury.
- Alterations in concentrations of ABA in roots, xylem sap and aerial plants parts from before development of dormancy and cold acclimation is initiated and until de-acclimation is accomplished. Following such a line it would be possible to determine whether there is a correlative relationship between ABA in different organs and hence whether/how ABA is transported between organs in cold acclimating plants.
- Identification of proteins, which are up-regulated during the cold season and determination of their function
- Determination of cold hardiness and associated adaptations in other *Hydrangea* species of intermediary hardiness compared to *H. macrophylla* and *H. paniculata,* to investigate whether there is a causal relationship between genetic relatedness and cold hardiness and associated adaptations

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Drought Adaptation in *Fuchsia magellanica* and Its Effect on Freezing Tolerance

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Abstract. **Fuchsia (*Fuchsia* L.) is a popular woody ornamental, but it is very susceptible to frost injury during winter. As drought stress may be used to enhance freezing tolerance in woody plants, the effects of different types of water deficit on growth, selected physiological traits, and freezing tolerance were examined in *Fuchsia magellanica* Lam. 'Riccartonii'. Drought responses were investigated after 6 weeks of pretreatment, where individual plants grown in a greenhouse under conditions of unrestricted water supply were compared with plants subjected to cyclic or continuous water deficit. After an additional 4 weeks of treatment at short day (10 h) and low temperature (8 °C day/4 °C night), freezing tolerance was examined. Both continuous and cyclic water deficit plants reduced water loss by reducing aboveground biomass and by efficient stomatal regulation. Continuous water deficit plants tended to adjust osmotically, while cyclic water deficit induced significantly higher xylem sap abscisic acid [$(ABA)_{xylem}$] and leaf proline concentrations and a lower leaf water potential (ψ_l) than continuous water deficit, indicating that *F. magellanica* responds differently to continuous water deficit and to fast drying associated with stress phases of cyclic water deficit. The root water potential (ψ_r) and $(ABA)_{xylem}$ were negatively linearly correlated, implying that increasing water deficiency stimulated formation of ABA in the roots. An inverse, curvilinear relation between $(ABA)_{xylem}$ and stomatal conductance (g_s) indicated that root-originated ABA might control g_s during mild water deficits. Neither cold-acclimating conditions alone nor combined with water deficit increased stem freezing tolerance, indicating that *F. magellanica* lacks cold-acclimation ability under the inductive conditions used in this study.**

Fuchsia magellanica is a prevalent woody ornamental in Denmark and in many other countries in the temperate zone. It is regarded as one of the hardiest outdoor *Fuchsia* species, but aboveground parts can be frost-injured or have dieback to the ground in winter and the new growth in spring, even on mature plants, is frost-tender. Plants that show dieback to the ground in winter are occasionally able to produce new shoots from belowground parts in spring, and frost-injured plants usually recover well but do not start growing quickly until late spring and flowering occurs late summer. To avoid frost injury and thereby substantial economic losses, Danish producers of fuchsia usually store *F. magellanica* at about –1 °C during winter. Increased freezing resistance would make it possible to over-winter *F. magellanica* outdoors and still obtain a high-quality product, and it would enhance the marketing potential of *F. magellanica* as a garden shrub. *F. magellanica* is native to the southern Andes (Berry et al., 2004), which have a temperate climate, with mean monthly temperatures between ≈2.4 and –2.8 °C in the coolest month (Wardle et al., 2001). However, according to Villalba et al. (2003), who reported that the mean temperature of the coolest month ranges between 0 and 4 °C, absolute minimum temperatures may be lower than –25 °C.

Strategies allowing plants to tolerate subzero temperatures are mainly of two types: freezing avoidance and freezing tolerance. Tissues displaying freezing avoidance escape frost injury by lowering the freezing point within the cells with antifreeze substances like sugars, alcohols, and proteins. Accumulation of antifreeze substances only lowers the freezing point by a few degrees and can be regarded as a shallow supercooling strategy that will not succeed in environments where prolonged, deep, subfreezing temperatures exist. Freezing avoidance is a resistance mechanism mainly used by freezing-sensitive plants. However, some freezing-sensitive plants cannot be frost-acclimated at all and are injured at very mild freezing temperatures. Freezing-tolerant plants survive freezing by letting ice form extracellularly, which is a process associated with cellular dehydration (Guy, 2003; Sakai and Larcher, 1987; Wisniewski et al., 2003). In many woody plant species, cold acclimation, or development of freezing tolerance, is regulated by the shortening of photoperiod and by declining temperature (Weiser, 1970; Welling et al., 2002). However, other triggers than low temperature and short photoperiod are known to influence development of freezing tolerance. Drought stress-induced increase in freezing tolerance has been reported for some woody plants from the Northern Hemisphere (Anisko and Lindstrom, 1996; Li et al., 2002; Rinne et al., 1998). The common denominator of drought and environmental triggers of cold acclimation is the promotion of cellular dehydration, and water deficit induces several responses similar to those changes observed in cold-acclimating woody plants. Among these are reduced growth (Li et al., 2005), reduced tissue water content (Anisko and Lindstrom, 1996), osmotic adjustment, defined as an increase in osmotic

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pressure of cell sap resulting from more organic and inorganic solute molecules per cell (Amundson et al., 1993; Munns, 1988; Rinne et al., 1998) and increased endogenous levels of ABA (Aasamaa et al., 2002; Guak and Fuchigami, 2001; Gusta et al., 2005; Welling et al., 1997).

Osmotic adjustment is associated with a lowering of the osmotic potential (ψ_S) inside the cell. Under cold conditions, this is believed to lower the freezing point of the cell, reducing the probability of ice crystal formation. Additionally accumulated organic molecules may protect cell membranes and vital macromolecules by maintaining their structural stability (Gusta et al., 2004; Li et al., 2004). Organic compounds often accumulating in cold-stressed woody plants include soluble carbohydrates, various proteins including dehydrins and other late-embryogenesis–abundant (LEA) proteins, glycine betaine, and proline (Arora et al., 1992; Lennartsson and Ögren, 2002; Rinne et al., 1998; Xin and Browse, 2000).

ABA, a well-known stress-inducible plant hormone, is suggested to mediate plant responses triggering cold acclimation and drought adaptation. ABA levels have been reported to increase in tissues of both herbaceous and woody plants subjected to drought or cold stresses (Li et al., 2002; Skriver and Mundy, 1990), and application of ABA can induce increasing freezing tolerance and drought tolerance (Guak and Fuchigami, 2001; Shinozaki and Yamaguchi-Shinozaki, 1996). There is a wealth of evidence demonstrating the involvement of ABA in root water availability perception, root-to-shoot communication regulating stomatal aperture and leaf growth, and altered gene expression in water deficit plants (Chaves et al., 2003; Davies et al., 2005; Loewenstein and Pallardy, 2002). In cold-acclimating plants, ABA is believed to be involved in photoperiod or temperature perception, water status alterations and osmoregulation, and in induction of cold-responsive genes (Rinne et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 1996).

Due to the similarities between plant responses to water deficit and to low temperatures, we hypothesized that freezing tolerance of *F. magellanica* may be increased by exposure to water deficit before cold. To our knowledge, no studies have been conducted investigating either effects of reduced water availability on the physiology of *F. magellanica* or its freezing tolerance. The objectives of the present study were to 1) determine effects of different applications of water deficit on growth and selected physiological traits in *F. magellanica* plants grown in controlled conditions and 2) determine if these effects were associated with changes in freezing tolerance. This was done by evaluating effects of continuous water deficit and cyclic water deficit on *F. magellanica* plants followed by short day and low temperature acclimation under controlled conditions. Freezing tolerance was determined in nonacclimated, fully watered plants and in acclimated plants either fully watered or subjected to continuous or cyclic water deficit.

Materials and Methods

Plant material and experimental treatments. In May 2005, cuttings of *F. magellanica* were stuck in plugs in sphagnum peat and covered by plastic under greenhouse conditions at 22 °C. After 5 weeks, rooted plants were potted in sphagnum peat in 0.67-L pots. The potted plants were grown under natural light conditions (17-h photoperiod) at 21 °C day and night with venting at 25 °C. When radiation reached 900 $W \cdot m^{-2}$ [equivalent to a photosynthetic photon flux density (*PPF*) of ≈1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$], insulating curtains were drawn. During production, plants were ebb- and flood-irrigated daily with a standard nutrient solution containing (in mM): 11.7 N, 0.8 P, 5.4 K, 3.5 Ca, 0.8 Mg, and 1.4 SO_4, at electrical conductivity (EC) 1.7 and pH 5.8. Micronutrients were added, making up 0.1‰ of the nutrient solution. The plants were cut back twice during production.

At onset of the experiment, 90 plants of uniform size were selected. Of these plants, 10 were randomly chosen and the pots were weighed daily to determine mean water consumption per plant. The soil-water potential (ψ) was determined by tensiometers (E-sensors, 0–1000 hPa; Tensio-Technik, Geisenheim, Germany) randomly positioned in three pots per treatment connected to a DT 505 data logger (Datataker Pty Ltd, Rowville, VIC, Australia). Below –50 kPa, the tensiometers were no longer reliable due to loss of contact between tensiometer and growing media, and treatments were based on gravimetric water loss. The remaining 80 plants were randomly arranged into three groups. Based on daily mean water consumption, control plants ($n = 40$) were top-watered daily with a volume of nutrient solution equal to 100% of evapotranspiration. The two treatment groups ($n = 20$) included two types of water availability treatments: continuous water deficit and cyclic water deficit.

At onset of the experiment, irrigation was withheld from continuous and cyclic water deficit plants until visible turgor loss. Thereafter, continuous water deficit plants were given an amount of nutrient solution equal to 40% of mean daily water consumption throughout the rest of the experimental period. The cyclic water deficit treatment was a repeating nonlethal cyclic water deficit treatment alternating between stress phases, where watering was withheld, and recovery phases where the plants received the same amount of water and nutrient solution as control plants for 5 d. Although water treatments were based on mean water consumption, at regular intervals all pots were weighed and weight adjustments were made on an individual-pot basis. In addition, three pots per treatment were weighed daily. Changes of daily pot weight throughout the experiment are shown in Fig. 1. In total control plants received ≈3.8 L of water per plant during the experiment, while continuous and cyclic water deficit plants received ≈1.4 and ≈1.8 L per plant, respectively. Pots were rotated daily to ensure a random distribution of growth conditions in the greenhouse.

After ≈6 weeks of treatment, plant-water relations were determined, five plants per treatment were harvested, and samples were collected for further analyses. During measurements, the cyclic water deficit plants were in a stress phase. After harvest, the remaining water deficit plants and half of the rest of the control plants were cold-acclimated in a climate chamber at 8 °C day/4 °C night temperature (LT), 60–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a daily photoperiod of 10 h (SD). Water deficit regimes were continued in the climate chamber, and hence the treatments applied in the climate chamber were control + SD + LT, constant water deficit + SD + LT, and cyclic water deficit + SD + LT. Another half of the control plants stayed in the greenhouse under a long day/warm temperature regime and served as nonacclimated control plants.

Stomatal conductance and plant-water relations measurements. Stomatal conductance to water vapor (g_s) of one of the third youngest fully expanded leaves was measured after ≈6 weeks of treatment with a CIRAS-2 IR gas analyzer

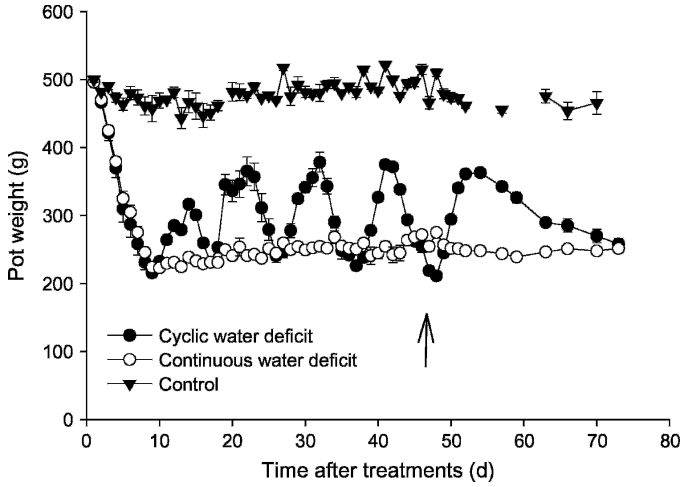

Fig. 1. Mean pot weight of *Fuchsia magellanica* plantlets during 6 weeks of pretreatment followed by 4 weeks of short-day and low-temperature (10 h; 8 °C day/4 °C night) acclimation. Plants were well-watered or subjected to continuous or cyclic water deficit during the entire period (means ± SE, $n = 3$). Arrow denotes when short day and low temperature acclimation was initiated.

(IRGA) equipped with a PLC6 Leaf Cuvette (PP Systems, Hertfordshire, U.K.). The humidity of the air stream was at 80% relative humidity (RH), and light was supplied from a light-emitting diode (LED) light source at a *PPF* of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Measurements were made between 1100 and 1400 HR, and readings were logged every 30 s until stable values of g_s were reached.

Midday leaf water potential (ψ_l) was measured with a pressure chamber (SKPM 1400; Skye Instruments Ltd, Llandrindod Wells, U.K.). Because of the short petioles in *F. magellanica*, it was impossible to seal a single leaf in the vessel. Consequently, a stem further up the plant with two to four pairs of leaves was sealed, and ψ_l was then recorded. Leaf osmotic potential (ψ_s) was determined on the same tissues after determination of ψ_l. The samples were wrapped tightly in aluminum foil, frozen in liquid nitrogen, and stored at –80 °C. After thawing, samples were placed in silicone tubes and sap was expressed by squeezing the tubes in a vice. The ψ_s of the sap was measured at 20 °C by psychrometers (C-52 sample chambers; Wescor, Logan, UT). Values of ψ_s were averages of two measurements from each sample. Leaf turgor pressure (ψ_p) was calculated as the difference between ψ_l and ψ_s.

Tissue analysis and plant biomass. At harvest, 1–2 g fresh weight (FW) of the fourth youngest fully expanded leaves of each plant were harvested and frozen in liquid nitrogen for determination of carbohydrates. Concentrations of hexose (glucose + fructose) and sucrose were determined by high-performance liquid chromatography (HPLC) (model 1047A; Hewlett Packard, Waldbronn, Germany) as described by Liu et al. (2004). Starch was determined in the remaining pellets after extraction of soluble sugars. The pellets were dried in a vacuum centrifuge, and the starch was gelatinized by boiling for 1 h with a thermostable amylase (Termamyl; Novo Nordisk, Glostrup, Denmark) in a 5 mM sodium dihydrogen phosphate buffer (pH 6.0). After centrifugation, the gelatinized starch in the supernatant was further hydrolyzed with amyloglucosidase (Roche Diagnostics, Basel, Switzerland) in 50 mM sodium acetate buffer and 15 mM magnesium chloride (pH 4.6) at 55 °C for 1 h. The extracts were purified by anion-exchange Sephadex QAE-A-25 (Pharmacia Biotech, Uppsala, Sweden) chromatography. The columns (1.0-mL volume) were pre-equilibrated with 0.5 mL of sodium formate and washed with 50 mL of 0.05 M sodium formate before sample application. The eluates were evaporated to dryness and redissolved in 0.5 mL of water, and the glucose concentration was analyzed on HPLC.

At the same time, leaves were sampled for quantification of proline concentrations. Between 250 and 500 mg FW of the second youngest fully expanded leaves of each plant was excised and frozen in liquid nitrogen. The samples were later analyzed using the acid–ninhydrin procedure described by Tamayo and Bonjoch (2001). In short, proline was determined colorimetrically by absorbance of the proline–ninhydrin product at 520 nm in a spectrophotometer using toluene as a solvent.

Leaf biomass was determined by recording the number and FW and dry weight (DW) of green, living leaves on five plants per treatment. Immediately after excision, the area of a subsample of leaves was determined using an area meter (LI-3000; Lambda Instruments Corp., Lincoln, NE). The area/FW ratios of the subsamples were used to calculate total leaf area of each plant. Average leaf size was determined by dividing total leaf area with total number of leaves. Specific leaf area (SLA) was calculated as total leaf area (square centimeters) per total DW of leaf biomass (grams).

Collection of xylem sap and determination of xylem sap (ABA). Potted plants were transferred to a Scholander-type pressure chamber and decapitated ≈5 cm above the soil surface. With the stem stump protruding outside the chamber, pressure was applied until the root water potential (ψ_r) was equalized. After determination of ψ_r, the cut surface was cleaned with pure water and dried with blotting paper, and the root system was gradually pressurized until the pressure equaled ψ_l. Before pressurizing the root system to ψ_l, a piece of silicon tube was placed on the protruding stem, leading exuded xylem sap into an Eppendorf vial wrapped with aluminum foil. Xylem sap was collected for 30–45 min and thereafter stored at –80 °C until further analysis. The ABA concentration in xylem sap was determined without purification by an ELISA enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRC MAC 252) according to Asch et al. (2001).

Determination of freezing tolerance. Freezing tolerance of stems was determined from four replicate samples per treatment after 6 weeks of treatment in the greenhouse followed by 4 weeks of treatment under cold-acclimating conditions. Freezing tolerance was determined at 10 test temperatures, ranging from 20 to –20 °C, by measuring the extent of freezing injury using the ion-leakage method. Four 2-cm-long pieces of internodal stem tissue were rinsed under cold running tap water for 30 s and then under running demineralized water for 15 s. After they were rinsed, the samples were placed in 70-mL test tubes and incubated in a controlled freezer. Few drops of demineralized water were added to each sample to initiate ice formation. The freezer cooled at a maximum rate of 5 °C per hour to 0 °C and subsequently at 2 °C per hour until the selected temperature was reached. The selected temperature was maintained for 2 h, after which time the samples were withdrawn and thawed at 4 °C. Ions were extracted with 35 mL of demineralized water for 20 h at room temperature and the EC was measured (EC_{frozen}) using an ION570 ISE meter with temperature-corrected display (Radiometer, Copenhagen, Denmark). After determination of the EC, the samples were autoclaved for

1 h to allow maximum leakage of ions. After autoclaving, the samples were allowed to cool to room temperature and the EC was measured again ($EC_{autoclave}$). The EC of demineralized water (EC_{water}) was measured to give the zero level of EC. Relative electrolyte leakage (REL) was calculated as $(EC_{frozen} - EC_{water}) \times 100/(EC_{autoclave} - EC_{water})$.

Even when aboveground parts of *F. magellanica* show dieback to the ground in winter, plants are occasionally able to produce new shoots from below ground parts in spring. Hence, to investigate whether *F. magellanica* root and stem freezing resistance varies or whether the apparent greater root freezing resistance is due to insulation by the soil, root freezing tolerance was also determined. Freezing tolerance was determined in February in roots of untreated pot-grown plants kept outdoors, shielded by a net, under natural cold-acclimating conditions. Due to the rather late date of investigation and thus risk of deacclimation, and as a result of an error in the programmable freezer which made it stop freezing when it reached –8 °C, root freezing tolerance was also determined in untreated 2-year-old commercially produced plants cold-acclimated outdoors during autumn and stored at –1 °C during winter. Peat was carefully removed from the roots by rinsing for 1 min under cold, running tap water. Subsequently remaining peat residues etc. were removed by hand, and roots were dip-rinsed in three beakers containing 0.2 L of demineralized water. Roots of plants kept outdoors were pooled from four plants per replicate, and samples of 100–300 mg consisting of 1–3 root pieces were prepared from roots <2 mm in diameter. Roots of plants kept in cold store were pooled from two plants per replicate and samples of 300–600 mg, consisting of one to two pieces of roots <2 mm in diameter were prepared. Five replicates from each set of plants were sealed in 70-mL test tubes with few drops of demineralized water and held at 4 °C as unfrozen control. To ensure initiation of ice crystal formation during freezing, the remaining samples were placed in 70-mL test tubes containing 35 mL of frozen, demineralized water. Subsequently, the samples were incubated in a programmable freezer. The rest of the analysis was performed in the same way as the analysis of stem freezing tolerance.

Data analysis. The effects of treatment on leaf biomass, plant-water relations, concentrations of carbohydrates and proline in leaves, and concentration of ABA in xylem sap were examined by the GLM procedure of SAS (SAS Institute, Cary, NC). Heterogeneities of variances within treatments were tested using Bartlett's test. When necessary, logarithmic transformations were performed to ensure homogeneity of variance. Differences between individual means were identified using Tukey's Studentized range (HSD) test at the 5% significance level. A linear relationship between ψ_r and $(ABA)_{xylem}$, determined on the same plants, was examined using Pearson's correlation coefficient, while a relation between $(ABA)_{xylem}$ and g_s was evaluated by a logarithmic function. The parameters of the curve were derived by the nonlinear least-squares iteration procedure (PROC NLIN), and the coefficient of determination (r^2) was calculated as 1 – SSE/CSS, where SSE is the residual sum of squares and CSS is the corrected total sum of squares.

Stem electrolyte leakage data were subjected to two-way analysis of variance (ANOVA) to determine the main effects of treatment and freezing temperature on REL. Tukey's HSD test was used for multiple comparisons of the effects of treatment and freezing temperature on REL. Stem REL data were arcsin square-root–transformed before analysis, but for clarity all data are presented as untransformed. A nonparametric Kruskall–Wallis test was also used to test differences in REL of stems between treatments and freezing temperatures. This test was done to supplement the results from the two-way ANOVA, as the assumption concerning similar variance was not fulfilled. Root electrolyte leakage data were analyzed using two-way ANOVA and Tukey's HSD test to determine main effects of freezing temperature and storage (outdoor vs. cold storage) and to separate individual means. Logarithmic transformations were performed to ensure homogeneity of variance, which was tested using Bartlett's test. To estimate the temperature representing 50% injury (LT_{50}), data for all four (stems) or five (roots) replicates per treatment were fitted by regression analysis (PROC NLIN), to the sigmoid function $y = y_0 + a/\{1 + \exp[-(x - x_0)/b]\}$, where y is REL with a value of y_0 at the lower asymptote, x is the temperature with a value of x_0 at the inflection point representing LT_{50}, and a and b are parameters. For roots of pot-grown plants kept outdoors a LT_{50} value could not be determined, because samples were only frozen to –8 °C, which made it impossible to fit data to the sigmoid function. For stems, differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap. To determine whether LT_{50} estimates differed between stems and roots, REL data for both stems and roots were fitted by regression analysis (PROC NLIN) to the sigmoid function $y = y_0 + a/\{1 + \exp[-(x - (x_0 + x_{0rod} \times \text{rod})/b)]\}$, where y, y_0, x, a, and b are the same parameters as described above and $x_0$rod is the difference between stem and root LT_{50} estimates. The difference in LT_{50} between the two tissue types was taken as significant if it differed significantly from 0.

To sum up, plants were initially pretreated (control, continuous water deficit, and cyclic water deficit) under a long-day/high-temperature regime in greenhouse. After 6 weeks of pretreatment, measurements and harvesting were performed. After harvesting, another set of pretreated plants was subjected to SD + LT in climate chamber for an additional 4 weeks, while the previous treatments were continued. Finally, stem freezing tolerance was determined. During the last 4 weeks, half of the control plants stayed in the greenhouse and served as non-acclimated controls.

Results

Plant biomass. Compared with control plants, total leaf fresh weight and total leaf area were significantly reduced by 27% and 41% in cyclic and continuous water-deficit plants, respectively (Fig. 2). Water-deficit treatments also significantly affected average leaf size, which was reduced from 1.42 cm^2 per leaf in control plants to an average of 1.16 cm^2 per leaf and 1.32 cm^2 per leaf in continuous water-deficit and cyclic water-deficit plants, respectively (Table 1). The mean number of leaves per plant tended to decrease nonsignificantly from 400 of control plants to ≈300 of both continuous and cyclic water-deficit plants (data not shown). Cyclic water deficit decreased SLA by 14% relative to control plants, whereas SLA of continuous water-deficit plants did not differ significantly from any of the other treatment groups (Table 1).

Stomatal conductance and plant-water relations. Both types of deficit irrigation significantly reduced g_s. Compared with control plants, g_s was reduced by ≈55% in both continuous and cyclic water-deficit plants (Table 1). Leaf water content

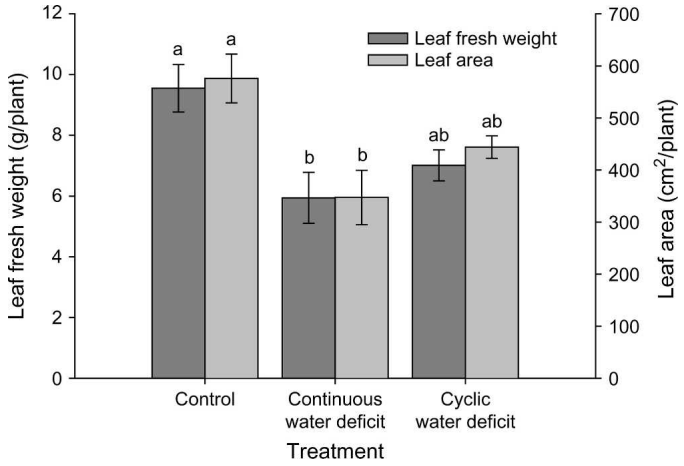

Fig. 2. Leaf area and leaf fresh weight of control, continuous water-deficit, and cyclic water-deficit *Fuchsia magellanica* plants after 6 weeks of treatment (mean ± SE, $n = 5$). Different letters above bars indicate significant differences ($P < 0.05$) among treatments according to Tukey's Studentized range (HSD) test.

was only significantly reduced by cyclic water deficit by 20% relative to control and continuous water-deficit plants (Table 1). That cyclic water deficit appeared more stressful than continuous water deficit was also apparent from decreases in ψ_r and ψ_l (Fig. 3). The root water potential did not differ significantly between control and continuous water-deficit plants but was significantly reduced, on average by 370%, in cyclic water-deficit plants. The variation in ψ_l between plants subjected to different treatments was less pronounced than the variation in ψ_r, but the tendency was the same. Neither continuous water deficit nor cyclic water deficit had any significant effects on leaf ψ_s and leaf ψ_p (data now shown). However, ψ_s tended to be lower in both stress treatments relative to control plants, whereas ψ_p tended to be higher and lower in continuous water-deficit and cyclic water-deficit plants, respectively, compared with control plants.

Carbohydrate and proline concentrations. The leaf starch concentration was lower in cyclic water-deficit plants compared with continuous water-deficit plants, but it did not differ significantly from the concentration in well-watered plants (Table 2). Leaf hexose (glucose + fructose) concentrations tended to be higher in water-deficit than in control plants, but due to a rather large variation within treatments, differences were not statistically significant. Sucrose concentrations were generally low and very inconsistent (data not shown). The proline concentration in leaves was significantly higher, by ≈30%, in cyclic water-deficit plants compared with the other treatments (Table 2).

Xylem sap (ABA). Enhanced levels of $(ABA)_{xylem}$ were observed in water-deficit plants. Compared with a mean concentration of 0.3 nmol mL^{-1} in control plants continuous water deficit and cyclic water deficit increased $(ABA)_{xylem}$ 2.5 and 10 times, respectively. The relationship between $(ABA)_{xylem}$ and g_s was inversely curvilinear and could be well described by a logarithmic function (Fig. 4, $P < 0.0001$). There was a significant linear relationship between ψ_r and $(ABA)_{xylem}$ across the water-deficit treatments (Fig. 5, $P < 0.0001$).

Freezing tolerance. When assessing differences in REL of stems between treatments and freezing temperatures by two-way ANOVA or Kruskal–Wallis tests, similar results were obtained. Both types of tests showed that REL of stems was significantly affected by the freezing temperature, whereas there was no difference in freezing tolerance between treatments, indicating that neither cold-acclimating conditions nor cold-acclimating conditions combined with different types of water deficit had any significant effect on freezing tolerance in stems of *F. magellanica*. REL increased rapidly below –4 °C and reached maximum at –8 °C. This is in accordance with both the Tukey and Mann–Whitney tests, showing that the lowest freezing test temperature inducing stem electrolyte rates, not significantly different from unfrozen samples, was –4 °C. Additionally, LT_{50} estimates were not different among treatments, varying between –5.2 and –6.7 °C (Table 3).

Root REL of cold-acclimated plants kept outdoors or in cold storage differed significantly at 4 °C but did not differ at other temperatures, indicating that plants kept outdoors had not started to deacclimate. Greater "control leakage" of roots of juvenile plants kept outdoors than from 2-year-old plants kept in cold storage could be due to their thinner structure, which may result in more injury and subsequent leakage during sample preparation. Tissue damage thresholds for roots, depicted by a rapid increase in REL, was less clear than in stems, as root REL was <40% at all temperatures, indicating that ions were retained in the tissue, although visually roots were clearly damaged at about –6 °C and below. However, the LT_{50} value for roots of cold-acclimated plants kept in cold store was significantly higher than the LT_{50} value for stems, indicating that *F. magellanica* roots are less freezing resistant than are stems (Table 3).

Discussion

The observed decrease in total leaf area and total leaf fresh weight in *F. magellanica* plants exposed to water deficit appeared to result mainly from a reduced average leaf size, which is a commonly observed response in woody plants subjected to drought stress (Bañon et al., 2003; Williams

Table 1. Average leaf size, specific leaf area, leaf water content, and stomatal conductance (g_s) of control, continuous water-deficit, and cyclic water-deficit *Fuchsia magellanica* plants after 6 weeks of treatment ($n = 5$).[z]

Treatment	Avg leaf size [mean ± SE (cm^2/leaf)]	Specific leaf area [mean ± SE ($cm^2 \cdot g^{-1}$ DW]	Water content, [mean ± SE ($g \cdot g^{-1}$ DW)]	Stomatal conductance (g_s) [mean ± SE ($mmol \cdot m^{-2} \cdot s^{-1}$)]
Control	1.42 ± 0.05 a	403 ± 7 a	5.69 ± 0.04 a	124 ± 9 a
Continuous water deficit	1.16 ± 0.06 b	373 ± 21 ab	5.40 ± 0.29 a	55 ± 12 b
Cyclic water deficit	1.32 ± 0.05 ab	346 ± 30 b	4.46 ± 0.82 b	53 ± 10 b

[z]Different letters within columns indicate significant differences ($P < 0.05$) between treatments according to Tukey's Studentized range (HSD) test.

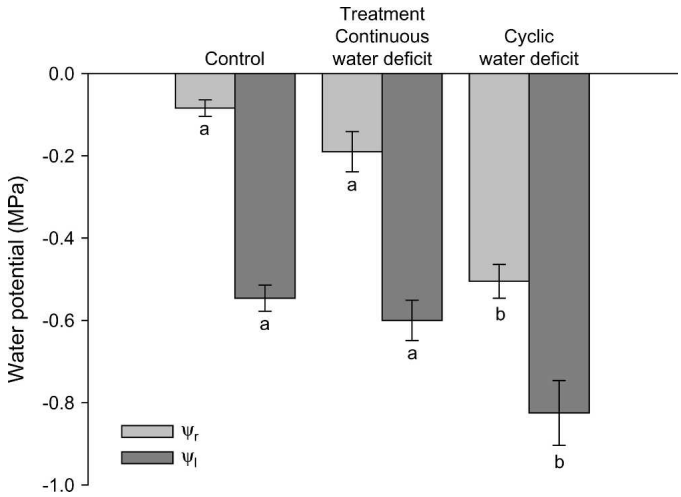

Fig. 3. Root water potential (ψ_r) and leaf water potential (ψ_l) in well-watered and continuous or cyclic water-deficit *Fuchsia magellanica* plants after 6 weeks of growth [mean ± SE, $n = 5$, except cyclic water deficit ($n = 4$)]. Different letters below bars indicate significant differences ($P < 0.05$) among treatments within organs according to Tukey's Studentized range (HSD) test.

et al., 1999). Reduced leaf area can be considered as a morphological adaptation to water stress to reduce transpiration and hence lower the consumption of water (Chaves et al., 2003). The average leaf number per plant tended to be lower in droughted plants, but differences were statistically insignificant, indicating that the activity of meristems was preserved during the stress period. This could be due to higher tolerance of cell division to dehydration in comparison with cell expansion, or alternatively it could be due to a better protection against dehydration of the meristem in comparison with older organs and tissues (Martìnez et al., 2004). A lower SLA in drought-stressed plants than in controls is also in accordance with results obtained with other species (Galmés et al., 2005; Yin et al., 2004) and may be a result of an increased investment in structural tissues, allowing increased resistance to unfavorable environmental conditions (Chaves et al., 2003).

Besides regulating leaf area, water-deficit plants also regulated water loss by inducing stomatal closure, which can be regarded as an avoidance mechanism reducing the rate at which water deficit develops (Chaves et al., 2003). Stomatal closure has been observed not only in water-deficit plants but also in deciduous leaves of cold-acclimating woody plants (Li et al., 2002, 2005).

Both continuous and cyclic water-deficit plants minimized water loss by closing stomata and reducing the leaf area, but cyclic water deficit had a much greater negative effect on the leaf water content and on ψ_l and ψ_r compared with continuous water deficit, suggesting that *F. magellanica* is more sensitive to stress phases of cyclic water deficit than to continuous water deficit. The leaf water content has been observed to decrease in woody plant species during cold acclimation (Li et al., 2002, 2005), and cellular dehydration is believed to be beneficial in plants subjected to cold, as tissues containing a large amount of free water are highly susceptible to formation of intracellular ice (Guy, 2003). Hence, reduced leaf water content in cyclic water-deficit plants may be advantageous in terms of cold resistance. Leaf ψ_s and ψ_p tended to decrease and increase, respectively, in continuous water-deficit plants compared with control plants, indicating that continuous water-deficit *F. magellanica* plants posses a modest ability to adjust osmotically. In contrast, the reduction in ψ_l and the nonsignificant reduction in ψ_s in cyclic water-deficit plants were probably induced by tissue dehydration since ψ_p and the leaf water content were also decreased. Lack of or limited capacity to adjust osmotically has also been observed in stems of other woody plants subjected to water deficit (Fan et al., 1994) and in leaves of drought-stressed *Rhamnus alaternus* L. (Banon et al., 2003). The apparent difference between continuous and cyclic water-deficit plants in ability to adjust osmotically may be ascribed to different rates of stress development, as osmotic adjustment is normally a slow process (Chaves et al., 2003). Hence, continuous water-deficit plants subjected to rather mild, prolonged drought may display modest osmotic adjustment, whereas a few days of fast droughting, as experienced by cyclic water-deficit plants, might not result in osmotic adjustment. Besides osmotic adjustment, turgor maintenance in continuous water-deficit plants may also be ascribed to adjustments in cell-wall elasticity, as observed in other woody plants species (Fan et al., 1994).

The foliar hexose concentration (glucose + fructose) tended to be higher in water-deficit plants, but differences were statistically insignificant, supporting the assumption concerning lack of or limited capacity for osmotic adjustment in water-deficit *F. magellanica*, because accumulation of sugars is generally considered to play an important role in osmotic adjustment in drought-stressed plants (Li and Li, 2005; Martìnez et al., 2004). According to Kingston-Smith et al. (1998), who compared leaf sucrose, starch, and hexose contents in source leaves of 13 plant species, *Fuchsia hybrida* L. accumulates much less sucrose and starch than other species, which may explain why we found very low and variable leaf sucrose concentrations. In cyclic water-deficit plants, the leaf starch content was reduced, but it seemed not to be an important source of hexose accumulation, although starch turnover can affect the foliar hexose pool (Kingston-Smith et al., 1998). However, as shown in apple (*Malus ×domestica* Borkh. 'Nagano Fuji'), water stress may primarily reduce foliar starch

Table 2. Concentrations of starch, hexose, and proline in leaves of *Fuchsia magellanica* after 6 weeks of growth subjected to control conditions, continuous water deficit, or cyclic water deficit [$n = 5$ except starch concentration, control ($n = 4$)].[z]

	Control	Continuous water deficit	Cyclic water deficit
Starch concn, dry weight yield [mean ± SE ($mg \cdot g^{-1}$ DW)]	15.43 ± 2.44 ab	18.73 ± 1.50 a	8.61 ± 1.79 b
Hexose concn, dry weight yield [mean ± SE ($mg \cdot g^{-1}$ DW)]	8.84 ± 5.29 a	17.26 ± 1.81 a	13.22 ± 7.12 a
Proline concn, fresh weight yield [mean ± SE ($\mu mol \cdot g^{-1}$ FW)]	0.68 ± 0.07 b	0.56 ± 0.02 b	0.88 ± 0.05 a

[z]Different letters within rows indicate significant differences between treatments ($P < 0.05$) according to Tukey's Studentized range (HSD) test.

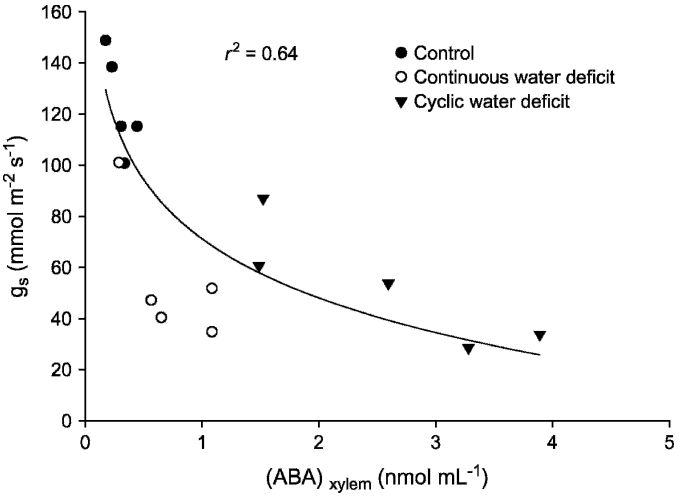

Fig. 4. Relationship between stomatal conductance (g_s) and xylem sap ABA concentrations [$(ABA)_{xylem}$] in control, continuous water-deficit, and cyclic water-deficit *Fuchsia magellanica* plants after 6 weeks of treatment (n = 15). r^2 significant at $P < 0.0001$ when data were fitted to a logarithmic function using the nonlinear least-squares iteration procedure, where r^2 was calculated as 1 – SSE/CSS where SSE is the residual sum of squares and CSS is the corrected total sum of squares.

Table 3. Freezing tolerance assessed as LT_{50} values (temperatures representing 50% injury) of stems and roots of *Fuchsia magellanica* plants.[z]

Plant organ	Treatment	LT_{50} [mean ± SE (°C)]
Stems	Control	–6.7 ± 1.7 aB
	Control + SD + LT	–5.7 ± 1.4 aB
	Continuous water deficit + SD + LT	–5.2 ± 0.4 aB
	Cyclic water deficit + SD + LT	–6.5 ± 0.3 aB
Roots	Kept in cold storage	–2.9 ± 0.4 A

[z]Stems of plantlets were tested after 6 weeks of control, continuous water-deficit, or cyclic water-deficit pretreatments and additionally 4 weeks of treatment under short-day [SD (10 h light)] and low-temperature [LT (8 °C day/4 °C night)] conditions. Roots of 2-year-old plants were tested after cold acclimation under natural conditions and cold storage at about –1 °C. LT_{50} are shown for four (stems) or five (roots) plants tested at 10 temperatures. Different lower-case letters within the column indicate significant differences between treatments within stems, and different upper-case letters within the column indicate a significant difference between tissue types. Differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap.

content via decreased synthesis due to altered partitioning of newly fixed carbon (Li and Li, 2005). Moreover, on the basis of the present results, it is not possible to rule out that *F. magellanica* might predominantly accumulate other soluble carbohydrates than hexose and sucrose during water deficit.

Constitutive proline levels in unstressed plants and proline accumulation during stress varies considerably in woody plants (Ain-Lhout et al., 2001; Martìnez et al., 2004; Matos et al., 2004). In the present experiment, the proline concentration increased significantly only in cyclic water-deficit plants, which were most severely stressed. This is in agreement with observations of Sánchez et al. (1998) and Yin et al. (2005), who reported that proline only accumulated in leaves of

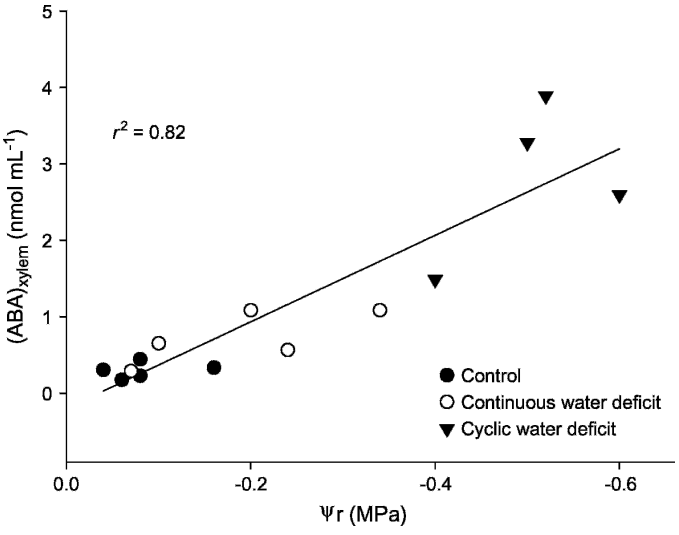

Fig. 5. Relationship between xylem sap ABA concentrations [$(ABA)_{xylem}$] and root water potentials (ψ_r) in control, continuous water-deficit, and cyclic water-deficit *Fuchsia magellanica* plants after 6 weeks of treatment (n = 14); r^2 significant at $P < 0.001$ according to Pearson's correlation coefficient.

different *Pisum sativum* L. cultivars after considerable drops in ψ_l and in severely drought-stressed *Populus kangdingensis* Wang et Tung. Considering the fairly low proline concentrations, the importance of proline as an osmolyte seems modest, but proline is confined to the cytoplasm (Aubert et al., 1999), which comprises only a small percent of the cell volume. Additionally, proline may modulate membrane permeability and ion uptake, protect protein structures, or function in redox-homeostasis (Rai, 2002).

ABA is thought to play an important role as a signal transducer in both water-stressed and cold-acclimating plants, and the present study shows that ABA is involved in drought-stress responses in *F. magellanica*. The observed xylem sap ABA concentrations were within the range reported for other woody species (Loewenstein and Pallardy, 2002; Soar et al., 2004), and increasing values in water-stressed plants are in accordance with earlier reports of an increased ABA concentration induced by drought in other plant species (Davies et al., 2005). Much higher $(ABA)_{xylem}$ in cyclic than in continuous water-deficit plants supports the assumption of *F. magellanica* being more sensitive to cyclic water deficit than to continuous water deficit. The strong linear correlation between $(ABA)_{xylem}$ and ψ_r indicates that *F. magellanica* is able to sense the soil-water availability and that increasing water deficiency stimulates formation of ABA in the roots, which is transported in the xylem sap. A similar relationship has been observed in other plants (Liu et al., 2005a, 2005b). The stomatal conductance (g_s) was closely correlated to $(ABA)_{xylem}$, and the curvilinear correlation indicates that, as in other woody and herbaceous plants (Liu et al., 2005a; Loewenstein and Pallardy, 2002; Soar et al., 2004), root-originated xylem sap ABA might control g_s during mild water deficits, whereas at severe water deficits, the dependency of g_s on $(ABA)_{xylem}$ is insignificant.

Cold-acclimating conditions (SD + LT) did not enhance freezing tolerance significantly in stems of *F. magellanica* compared with plants grown in the greenhouse under a long-day/high-temperature regime. The apparent lack of increased freezing tolerance indicates that *F. magellanica* has not

developed effective acclamatory responses to the cold-acclimating conditions used in this study. According to Sakai and Larcher (1987), freezing temperatures down to about –5 °C are common in freezing-sensitive species due to accumulated solutes inside cells, which might explain the minor capacity of *F. magellanica* to withstand freezing temperatures. Alternatively, *F. magellanica*'s cold-acclimation potential may be <2 °C, which was the freezing temperature interval used in the present study. In the tropical woody plant species *Nothofagus nitida* (Phil.) Krasser exhibiting freezing avoidance, a very modest increase in leaf freezing resistance (≈2 °C) was observed during cold acclimation (Reyes-Díaz et al., 2005). Hence, the acclimation capacity of *F. magellanica* may be marginal and therefore not apparent in the present study. Otherwise *F. magellanica* may require lower temperatures to initiate cold acclimation, as taxa acclimate differentially within a given temperature range (Weiser, 1970).

Water deficit has been shown to enhance freezing tolerance in some woody plant species in the Northern Hemisphere, although the increase may be small (Anisko and Lindstrom, 1996; Li et al., 2002; Rinne et al., 1998). However, our results showed that water deficit applied before and during cold-acclimating conditions did not influence freezing tolerance of *F. magellanica* stems. Because accumulation of solutes (e.g., soluble carbohydrates and proline) is essential in plants displaying freezing avoidance (Rada et al., 2001; Reyes-Díaz et al., 2005), the reason why water deficit did not enhance freezing resistance of *F. magellanica* may be ascribed to lack of or very modest accumulation of soluble carbohydrates during drought stress. However, if *F. magellanica* has not developed acclamatory responses to freezing, it may be unfeasible to improve freezing resistance via accumulation of soluble sugars. In that case, it is also unlikely that ABA production triggered by water deficit influences freezing tolerance. Although cyclic water deficit induced leaf dehydration, it did not improve freezing tolerance of stems. Hence, stems may not have been dehydrated or tissue dehydration alone is insufficient to increase freezing tolerance. Freezing tolerance of *F. magellanica* roots was lower than stem freezing tolerance. It is commonly observed in woody plants that roots seldom attain the same degree of freezing resistance as aboveground parts (Ryyppö et al., 1998; Sakai and Larcher, 1987; Stattin and Lindström, 1999), demonstrating that the frequent ability of *F. magellanica* to produce new shoots after killing of stems presumably is due to the lower impact of frost action in the soil.

In conclusion, the present findings indicate that *F. magellanica*, under both continuous and cyclic water deficit, postpones dehydration by modulating leaf area and closing stomata, whereas only continuous water-deficit plants develop modest leaf osmotic adjustment. Water deficiency stimulates formation of ABA in the roots, which, at mild water deficit, probably regulates stomatal closure. *F. magellanica* stems and roots are most likely freezing sensitive, and due to *F. magellanica*'s lack of acclamatory mechanisms to cold-acclimating conditions used in this study or since drought stress is not associated with alterations of plant-water relations beneficial in terms of freezing resistance, water deficit applied before and during cold acclimation does not improve freezing tolerance. In terms of management practice, it is therefore unlikely that successful over-wintering of *F. magellanica* outdoors in Denmark and in similar climatic areas can be obtained as a result of drought treatments before cold stress.

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Effects of chilling temperatures and short photoperiod on PSII function, sugar concentrations and xylem sap ABA concentrations in two *Hydrangea* species

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ABSTRACT

Cold injuries are frequently seen in *Hydrangea macrophylla* but not in *Hydrangea paniculata*. This may be ascribed to different levels of hardiness in the non-acclimated and the acclimated state, and to differences in responses to short day (SD) and low temperature (LT) and hence in the ability to cold acclimate. In this study *H. macrophylla* ssp. *macrophylla* (Thunb.) Ser. 'Blaumeise' and *H. paniculata* Sieb. 'Kyushu' were exposed to short photoperiod (10-h) and 4 °C in controlled conditions for 25 days, with measurements and samplings carried out at regular intervals. Chlorophyll fluorescence measurements revealed significant alterations in O–J–I–P fluorescence kinetics and decreases in the photochemical efficiency of photosystem II in stressed plants, followed by less chlorophyll contents. Perturbations of the photosynthetic apparatus were relatively greater in *H. macrophylla* than in *H. paniculata*. Likewise, induction of a transient increase in xylem sap abscisic acid concentrations ($[ABA]_{xylem}$) and accumulation of soluble sugars in leaves and stems were different in the two species. Stem cold hardiness in the non-acclimated state did not differ between *H. macrophylla* and *H. paniculata*, indicating equal sensitivity to sudden temperature drops in the growing season. Despite adaptive responses induced by the treatment neither species developed increased stem cold hardiness, suggesting that cold acclimation in *Hydrangea* may require exposure to temperatures below ca. 4 °C.

1. Introduction

Two major components of successful performance of woody perennials in temperate climates are hardiness in the non-acclimated state ('constitutive' level of hardiness) and capacity to cold acclimate. Cold acclimation is induced by shortening photoperiod and declining temperature, and involves several physiological and biochemical changes, whereby plants become increasingly tolerant to subsequent freezing temperatures [1,2]. The level of hardiness in the non-acclimated state is essential in the autumn and early spring, when frost incidents may occur while plants are in a growth stage. Cold acclimation is essential to successfully withstand the first fall frost episodes. Although chilling temperatures (~12–0 °C) are implicated in induction of cold acclimation their occurrence are not strictly associated with the annual cycle of cold hardiness, but may occur throughout most of the year.

Cold hardiness in the non-acclimated state and responses to SD and LT have been shown to vary among species, ecotypes and sexes indicating genetic variation for constitutive cold hardiness and cold acclimation ability [3–7]. Adaptive responses to SD and LT include alterations in tissue concentrations of abscisic acid (ABA), induced losses of photochemical efficiency and altered carbohydrate metabolism. ABA is a well-known stress-inducible phytohormone and growth inhibitor, which increases in chilling sensitive and chilling tolerant annual plants [8,9] and in overwintering woody plants [5,10] exposed to SD and/or LT conditions, indicating an important role for ABA in the acquisition of chilling tolerance and/or cold acclimation.

Stress-induced perturbations in the photosynthetic apparatus measured during LT conditions by means of chlorophyll (Chl) *a* fluorescence has frequently been reported, also as a rapid screening method to asses chilling tolerance [11–13]. Alterations in Chl *a* fluorescence have mainly been studied in evergreen woody plants and herbaceous winter annuals, but the maximum quantum efficiency of photosystem II (F_v/F_m) has also been shown to decrease in deciduous leaves of woody perennials subjected to environmentally controlled chilling temperatures [14]. Most studies have used F_v/F_m, with a theoretical value of 0.83 in non-stressed plants, to monitor the physiological status of the photosynthetic apparatus, but F_v/F_m is just one of a number of parameters which can be derived from the Chl *a* fluorescence transient. Based on the fast rise in O–J–I–P fluorescence Strasser

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and Strasser [15] developed a so-called JIP test, which calculates a range of different parameters related to energy and electron fluxes in photosystem II (PSII), including the performance index (PI_{ABS}).

Exposure of plants to LT often results in accumulation of carbohydrates, as the photosynthetic energy capture is reduced to a lesser degree than the metabolic utilization processes [16]. Soluble sugars accumulating as a response to LT can play multiple roles. They can act as an immediate energy source upon stress removal and as osmoprotectants stabilizing cellular membranes and maintaining turgor [2,17]. In cold acclimating plants sucrose, glucose and fructose are probably the most studied soluble carbohydrates in the early course of cold hardening, whereas the raffinose family of oligosaccharides (raffinose and stachyose) has been associated with later, season-long freezing tolerance in several woody plants [18–20].

Hydrangea's are very popular ornamentals, widely used and commercially important in landscape gardening. Especially the species *H. macrophylla* ssp. *macrophylla* (Thunb.) Ser. is popular due to its attractive flower heads. In Denmark and other countries of the temperate zone it is a common problem that current year shoots of *H. macrophylla* are frost killed or injured during winter. As flower buds of most *H. macrophylla* varieties are formed during the fall and over-winter on dormant stems, flowering will only occur the following year if terminal and/or lateral flower buds are present and undamaged. Cold injuries may be ascribed to early fall frost, low minimum temperatures mid-winter or frost episodes in late winter and spring, since *H. macrophylla* is considered to acclimate late in fall and de-acclimate early in spring and in a number of cultivars maximum stem hardiness is limited to −18 °C–(−24 °C) [21]. *H. paniculata* Sieb. is hardly ever cold injured in Denmark and its maximum cold hardiness is considerably greater than *H. macrophylla*'s. Freezing tolerance of well-hardened stems of *Hydrangea paniculata* 'Grandiflora' clones of different origin, determined as the temperature representing 50% injury (LT_{50}), is −36 to −37 °C [22].

In addition to greater mid-winter hardiness, we hypothesized that the frequent cold injuries encountered in *H. macrophylla* but not in *H. paniculata* may be ascribed to different levels of cold hardiness in the non-acclimated state and more pronounced physiological adaptations to SD and LT. In *H. paniculata* we expected greater alterations in ABA and faster and/or greater accumulation of soluble carbohydrates and other protective substances, and in *H. macrophylla* we expected greater alternations in Chl *a* fluorescence of deciduous leaves in response to SD and LT. Hence, the present study was conducted to (1) determine the constitutive level of cold hardiness in *H. macrophylla* and *H. paniculata* and to identify physiological responses of the two species to a period of short days and low, non-freezing temperatures, and (2) determine whether differences in adaptive responses to SD and LT conditions may be related to differences in the capacity to cold acclimate in *H. paniculata* compared with *H. macrophylla*.

2. Materials and methods

2.1. Plant material and treatments

The experiment was carried out using 2-year old vegetatively propagated commercially produced *H. macrophylla* ssp. *macrophylla* (Thunb.) Ser. 'Blaumeise' and *H. paniculata* Sieb. 'Kyushu' plants grown in 3.5-L pots containing sphagnum peat. Prior to the experiment, in the beginning of April, plants were forced in a greenhouse at 24 ± 2 °C day and night with venting at 26 °C and a light:dark cycle of 18 h:6 h. Supplementary light was turned on when the photosynthetic photon flux density (PPF) was less than 70 μmol m^2 s^{-1} and switched off when the radiation exceeded 90 μmol m^2 s^{-1}. When the radiation reached 1600 μmol m^2 s^{-1} curtains were drawn. Plants were ebb and flood irrigated daily with a standard nutrient solution prepared from tap water containing (in mM): 12.2N–0.5P–4.3K–4.0Ca–0.7Mg–1.7SO_4, at EC 1.96 pH 6.0. Micronutrients were added making up 0.1‰ of the nutrient solution.

From 8 June to 15 June batches of plants of uniform size grown in the greenhouse were pre-acclimated in growth chambers at 20 ± 2 °C day and night, 18-h photoperiod and a PPF of 50–150 μmol m^{-2} s^{-1}. In growth chambers plants were drip-irrigated with a standard nutrient solution (Pioner NPK Macro 14-3-24, Brøste, Denmark) prepared from tap water containing (in mM): 10.3N–0.9P–5.9K–2.5Ca–2.2Mg–2.0SO_4 at EC 1.5, pH 5.5. Micronutrients (Pioner Micro, Brøste, Denmark) were added making up 0.125‰ of the nutrient solution. At onset of the experiment, two treatments were applied. One group of plants was exposed to LT (4 °C day:night) and SD (10-h), and the other group of plants was subjected to control temperatures (20 °C day:night) and a long day (18-h) and served as controls. To avoid shock reactions the temperature and the photoperiod in the SD and LT treatment were gradually lowered in the course of 3 days. The experiment lasted 25 days, where day 0 is defined as the day when the temperature and the photoperiod in the SD and LT treatment started to be lowered. Consequently, days 0–3 was the time period where the temperature and photoperiod in the SD and LT treatment were gradually lowered to the predetermined treatment levels. Non-destructive measurements, including shoot elongation growth, chlorophyll fluorescence and chlorophyll content, were performed on days 0, 3, 7, 10, 14, 16 and 21. Destructive sampling for determination of $[ABA]_{xylem}$ took place on days 1, 4, 8, 11, 18 and 22, while harvesting of leaves and stems for determination of soluble carbohydrates only took place on days 1, 11 and 22. Stem freezing tolerance was determined approximately once every week at days-2, 5, 12, 18 and 25. At each time (treatment duration) separately elongation growth was measured on five replicates per treatment and per species. All other measurements and sample collections included five and three replicates per species of stressed and control plants, respectively.

2.2. Shoot elongation growth

Shoot elongation growth was determined with a ruler measuring the distance between the shoot tip and a reference mark made between the tip and the first node.

2.3. Chlorophyll fluorescence and chlorophyll content

Chlorophyll fluorescence measurements were made on three of the third youngest fully expanded leaves per replicate. Fluorescence emissions were measured using a portable chlorophyll fluorometer (Handy-PEA, Hansatech Instruments, King's Lynn, Norfolk, UK). The chlorophyll fluorometer emits light of 650 nm wavelength with an intensity of 2500 μmol m^{-2} s^{-1} for 10 s. Measurements were carried out on plants dark adapted for 30 min to ensure an initial zero photochemical activity and CO_2 fixation state. During light illumination the Chl *a* fluorescence intensity in dark-adapted leaves rises rapidly from an initial minimal level, F_0 (the O step), to the maximal level, F_m (P step). Two intermediary steps designated as J and I usually appear at ca. 2 ms and 30 ms, respectively, hence the notation O–J–I–P for the fast rise of the fluorescence transient. The O–J–I–P transients obtained by the BIOLYZER program [23] were visually examined for the effects of treatment and time and the performance index (PI_{ABS}) was calculated using the JIP test. The PI_{ABS} is a multiparametric expression combining the three main functional steps of photosynthetic activity by a PSII reaction centre complex, i.e. light energy

Table 1
Summary of technical data of the O–J–I–P curves and the selected JIP-test parameters used in this study

Technical fluorescence parameters	
F_0	$F_{50\ \mu s}$, fluorescence intensity at the O-step (at 50 μs)
$F_{100\ \mu s}$	Fluorescence intensity at 100 μs
$F_{300\ \mu s}$	Fluorescence intensity at 300 μs
F_J	Fluorescence intensity at the J-step (at 2 ms)
F_I	Fluorescence intensity at the I-step (at 30 ms)
F_m	Maximal fluorescence intensity at the P-step
V_J	Relative variable fluorescence at the J-step = $(F_J - F_0)/(F_m - F_0)$
Quantum efficiencies or flux ratios	
$\varphi_{Po} = TR_o/ABS$	Trapping probability or quantum yield efficiency. Expresses the probability that an absorbed photon will be trapped by the PSII reaction centre. $\varphi_{Po} = (F_m - F_0)/F_m = F_v/F_m$
$\psi_o = ET_o/TR_o$	Expresses the probability that a photon trapped by the PSII reaction centre enters the electron transport chain. $\psi_0 = 1 - V_J$
Reaction centres per chlorophyll	
$RC/ABS = (RC/TR_o) \times (TR_o/ABS)$	Expresses the density of operative photosystems. $RC/ABS = [(F_J - F_0)/4(F_{300\ \mu s} - F_0)] \times (F_v/F_m)$
Performance index	
PI_{ABS}	Multi-parametric expression of the three independent steps contributing to photosynthesis; absorption of light energy, trapping of excitation energy and conversion of excitation energy to electron transport. $PI_{ABS} = (RC/ABS) \times [(\varphi_{Po}/(1 - \varphi_{Po})] \times [\psi_o/(1 - \psi_o)]$

ABS, absorption energy flux; ET, flux of electrons from Q_A into the electron transport chain; RC, reaction centre; TR, energy flux for trapping.

absorption, trapping of excitation energy and the conversion of trapped energy to electron transport. Performance of the JIP test takes into consideration maximal fluorescence (F_m), fluorescence intensity at 50 μm (considered as F_0), fluorescence intensity at 300 μs ($F_{300\ \mu s}$) and the fluorescence intensity at 2 ms (the J step) denoted as F_J. Table 1 summarizes the fluorescence parameters extracted from the O–J–I–P transients and the selection of JIP-test parameters used in this study. For a detailed description of the relationship between the O–J–I–P fluorescence transient and the PI_{ABS} see Strauss et al. [24,25].

Chlorophyll contents were estimated using an optical leaf-clip (ADC:OSI CCM 200 Chlorophyll Content Meter, Hoddesdon, Herts, England), as chlorophyll concentrations, chlorophyll meter readings (SPAD values) and nitrogen concentrations are strongly correlated [26]. Three of the third youngest fully expanded leaves per replicate were measured, with three measurements being performed on each leaf.

2.4. Soluble carbohydrate analysis

Approximately 3 g FW of the third youngest fully expanded leaves and pieces of stems in parallel to the third youngest leaves were harvested and frozen in liquid nitrogen for determination of carbohydrates. Hexose (glucose + fructose) and sucrose was determined using high-performance liquid chromatography (HPLC, Hewlett Pacard 1047A, Waldbronn, Germany) as described by Liu et al. [27].

2.5. Collection of xylem sap and determination of xylem sap [ABA]

Before collection of xylem sap the leaf water potential (Ψ_l) was estimated by measuring Ψ_l of one of the first or second youngest fully expanded leaves with a pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). To collect xylem sap potted plants were transferred to a Scholander-type pressure chamber and decapitated approximately 5 cm above the soil surface. With the stem stump protruding outside the chamber, pressure was applied until the root water potential (Ψ_r) was equalised. After determination of the root water potential the cut surface was cleaned with pure water, dried with blotting paper and the root system was gradually pressurised until the pressure equalled Ψ_l. Exuding xylem sap was collected into an Eppendorf vial using a pipette. Xylem sap was collected for 5–20 min and thereafter stored at −80 °C until further analysis. The ABA concentration in xylem sap was determined without purification by an enzyme linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRC MAC 252) according to Asch et al. [28].

2.6. Determination of freezing tolerance

Freezing tolerance of current-year stems was determined at five test temperatures at 5 °C intervals, by measuring the extent of freezing injury by a slightly modified version of an electrolyte leakage method previously described for other woody plants [29]. One (*H. macrophylla*) or two (*H. paniculata*) 3-cm-long pieces of internodal stem tissue were rinsed under cold running tap water for 15 s and then under cold running demineralised water for 15 s. After rinsing the samples were placed in 70-mL test tubes and incubated in a controlled freezer. Few drops of demineralised water were added to each sample to initiate ice formation. The freezer cooled at a rate of maximum 5 °C per hour to 0 °C and subsequently at 2 °C per hour until the selected temperature was reached. The selected temperature was maintained for 2 h, where after the samples were withdrawn and thawed at 4 °C. Ions were extracted with 35 mL demineralised water for 20 h at room temperature and the electrical conductivity measured (EC_{frozen}) using a ION570 ISE-meter with temperature-corrected display (Radiometer, Copenhagen, Denmark). After determination of the electrical conductivity the samples were autoclaved for 1 h to allow maximum leakage of ions. After autoclaving the samples were allowed to cool to room temperature and the electrical conductivity was measured again ($EC_{autoclave}$). The electrical conductivity of demineralised water (EC_{water}) was measured to give the zero level of electrical conductivity. Relative electrolyte leakage (REL) was calculated as $REL = (EC_{frozen} - EC_{water}) \times 100/(EC_{autoclave} - EC_{water})$.

2.7. Data analysis

Data of shoot elongation rates, chlorophyll fluorescence (F_v/F_m and PI_{ABS}), chlorophyll contents, concentrations of soluble carbohydrates in leaves and stems and concentrations of ABA in xylem sap were analysed by a three-way analysis of variance (PROC GLM of SAS, SAS Institute, Cary, NC, USA). Main effects were considered to be treatment, species and time (treatment duration), as well as their interactions. Heterogeneities of variance within treatments and species at each time separately were tested using

Bartlett's Test. When necessary data were log-transformed to ensure homogeneity of variance, but for clarity all data are presented as untransformed. Differences between individual means were identified using the Tukey–Kramer test at the 5% significance level. As the assumption concerning homogeneity of variance was not fulfilled in the case of shoot elongation rates and F_v/F_m, those data was also tested using a non-parametric Kruskall–Wallis test to determine differences between treatments, species and time. Three-way ANOVA and Kruskall–Wallis tests gave similar results when assessing effects of different treatments, species and treatment durations on F_v/F_m and only the results of the ANOVA are described. However, the results of the ANOVA and Kruskall–Wallis tests differed when examining differences in stem elongation rates, in that case only the results of the Kruskall–Wallis tests are described.

For stem electrolyte leakage data, temperature was considered as another source of variation. According to both a four-way analysis of variance and Kruskall–Wallis tests stem REL was significantly affected by treatment, time and temperature, whereas it did not differ between species. Hence, for each time separately REL data were pooled across species and LT_{50} values assessed as temperatures representing 50% REL. LT_{50} values were estimated by fitting data for all six or ten replicates per treatment by regression analysis (PROC NLIN of SAS) to the sigmoid function $REL = REL_{min} + (REL_{max} - REL_{min})/(1 + \exp(c \times (d - T)))$, where REL_{min} is the base line of REL, REL_{max} is the maximum REL, c is the slope of the function at the inflection point d and T is the treatment temperature. The temperature (d) at the inflection point was used as LT_{50} [30]. Differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap.

3. Results

3.1. Shoot elongation growth

Shoot elongation growth of both *H. macrophylla* and *H. paniculata* ceased immediately under SD and LT, and *H. paniculata* presented significantly higher elongation rates than *H. macrophylla* under both control and SD and LT conditions (Fig. 1, Table 2). The daily elongation rate of control plants was 5.6 and 10.2 mm day^{-1} in *H. macrophylla* and *H. paniculata*, respectively, while the equivalent values of stressed plants were 0.5 and 1.1 mm day^{-1}.

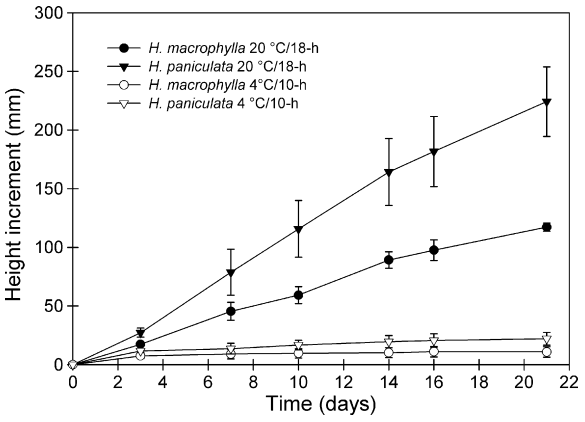

Fig. 1. Shoot elongation in *Hydrangea macrophylla* and *Hydrangea paniculata* exposed to 20 °C/18-h photoperiod or 4 °C/10-h photoperiod for 21 days. Values are mean ± S.E. of four to five replicates.

3.2. Chlorophyll fluorescence and chlorophyll content

Table 3 summarizes the extracted averages of the technical fluorescence parameters for each species, treatment and day, which were used to depict the O–J–I–P fluorescence transient and to calculate the JIP-test parameters. Excitation of dark-adapted leaves with a saturated light pulse induced typical O–J–I–P fluorescence transients in control plants, although at day 21 the fluorescence intensities were reduced in both species (Fig. 2). In SD- and LT-treated plants the shape of the O–J–I–P transients differed from those recorded in control plants, especially at the J and P steps. In both species low-temperature stress resulted in a large increase in the fluorescence intensity at 2 ms at day 3, and in *H. paniculata* this was a general trend throughout the experiment except at day 21. A reducing effect of LT on F_m appeared at day 7, where after F_m continued to decrease in a duration dependent manner. Values of both F_v/F_m and the PI_{ABS} were significantly affected by species and the interaction between treatment and time. F_v/F_m was additionally affected by the interaction between treatment and species (Table 2). F_v/F_m and the PI_{ABS} of control plants fluctuated around average values of 0.81 and 0.82 and average values of 37 and 28 for *H. macrophylla* and *H. paniculata*, respectively, and did not vary significantly over time (Fig. 3). Initially the F_v/F_m ratio of SD- and LT-treated plants was similar to

Table 2
H-values and significance of Kruskall–Wallis tests of shoot elongation rates and F-values and significance of a three-way ANOVA of chlorophyll fluorescence ratios (F_v/F_m), performance indexes (PI_{ABS}), chlorophyll contents, xylem sap ABA concentrations ($[ABA]_{xylem}$) and leaf and stem concentrations of sucrose ([Sucrose]) and hexose ([Hexose]) for *H. macrophylla* and *H. paniculata* grown under control conditions (20 °C/18-h) or short day and low temperature conditions (4 °C/10-h) for different treatment durations

Parameter	Main factors			Interactions			
	Treatment	Species	Time	Treatment × species	Treatment × time	Species × time	Treatment × species × time
Shoot elongation	78.11***	7.07**	5.01 ns				
d.f.	1	1	5/6	1	5/6	5/6	5/6
F_v/F_m	257.88***	24.87***	26.00***	9.17**	16.28***	0.42 ns	0.51 ns
PI_{ABS}	30.27***	678.68***	55.03***	1.13 ns	35.01***	0.36 ns	0.89 ns
Chlorophyll content	112.48***	286.71***	12.28***	11.82***	4.19**	2.30*	0.35 ns
$[ABA]_{xylem}$	4.89*	6.74*	0.90 ns	1.21 ns	1.29 ns	0.25 ns	0.61 ns
d.f.	1	1	2	1	2	2	2
$[Sucrose]_{leaves}$	33.38***	61.31***	39.52***	0.15 ns	6.12**	1.54 ns	0.31 ns
$[Sucrose]_{stems}$	31.69***	12.18**	10.03***	9.43**	13.01***	2.20 ns	0.41 ns
$[Hexose]_{leaves}$	82.24***	0.51 ns	3.06 ns	0.28 ns	6.02**	0.46 ns	1.49 ns
$[Hexose]_{stems}$	33.69***	93.61***	1.75 ns	18.73***	6.09**	2.97 ns	2.15 ns

The degrees of freedom (d.f.) for the main factor time were 6 for F_v/F_m, PI_{ABS} and chlorophyll contents and 5 for $[ABA]_{xylem}$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
Summary of the extracted averages of the technical fluorescence parameters of the O–J–I–P curves for *H. macrophylla* and *H. paniculata* exposed to 20 °C/18-h photoperiod or 4 °C/10-h photoperiod

Treatment	Time (days)	F_0	$F_{100\ \mu s}$	$F_{300\ \mu s}$	F_J	F_I	F_m
H. macrophylla, 20 °C/18-h	0	239	259	354	614	974	1200
	3	275	298	432	729	1147	1340
	7	252	274	379	654	1022	1238
	10	265	287	387	647	997	1189
	14	241	262	356	616	937	1158
	16	244	262	351	611	964	1165
	21	220	236	314	531	735	892
H. paniculata, 20 °C/18-h	0	231	258	383	667	934	1167
	3	226	254	401	657	949	1122
	7	211	233	336	584	798	1028
	10	246	276	418	706	1021	1175
	14	207	228	332	591	842	1046
	16	203	223	319	572	825	1022
	21	180	194	261	456	653	819
H. macrophylla, 4 °C/10-h	0	258	280	389	694	1058	1286
	3	306	331	460	847	1036	1288
	7	322	342	434	710	823	1007
	10	321	342	440	729	844	997
	14	317	338	427	685	794	954
	16	320	342	429	659	762	881
	21	282	297	362	564	659	746
H. paniculata, 4 °C/10-h	0	213	239	364	656	943	1146
	3	239	267	405	761	910	1098
	7	270	297	416	696	784	904
	10	270	299	433	733	829	929
	14	265	292	409	662	740	844
	16	271	302	427	668	743	812
	21	239	261	354	572	642	692

Values represent the average of 15 measurements (obtained from five plants per species) for short day and low temperature-treated plants and nine measurements (obtained from three plants per species) for control plants, and were recorded at regular intervals (days 0, 3, 7, 10, 14, 16 and 21).

that of control plants, but it soon started to decline in both species, but significantly more in *H. macrophylla* than in *H. paniculata*. From day 7 and onwards the F_v/F_m ratios of SD- and LT-treated plants were consistently lower than the ratios of control plants in both species. Low temperature conditions induced a relatively larger and faster decrease in the PI_{ABS} than in F_v/F_m, significant from day 3 and onwards. Irrespective of treatment PI_{ABS} values were lower in *H. paniculata* than in *H. macrophylla* and contrary to F_v/F_m the PI_{ABS}

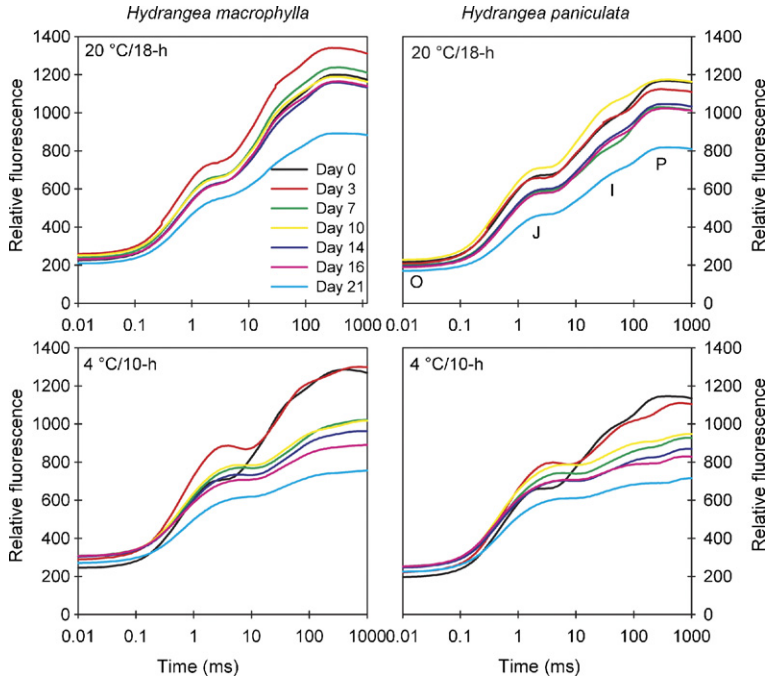

Fig. 2. Shape of the O–J–I–P transients recorded in leaves of *H. macrophylla* and *H. paniculata* exposed to 20 °C/18-h photoperiod or 4 °C/10-h photoperiod. Transients represent the average of 15 measurements (obtained from five plants per species) for short day and low temperature-treated plants and nine measurements (obtained from three plants per species) for control plants, and were recorded at regular intervals (days 0, 3, 7, 10, 14, 16 and 21). The position of the O, J, I and P steps are indicated in the right top figure.

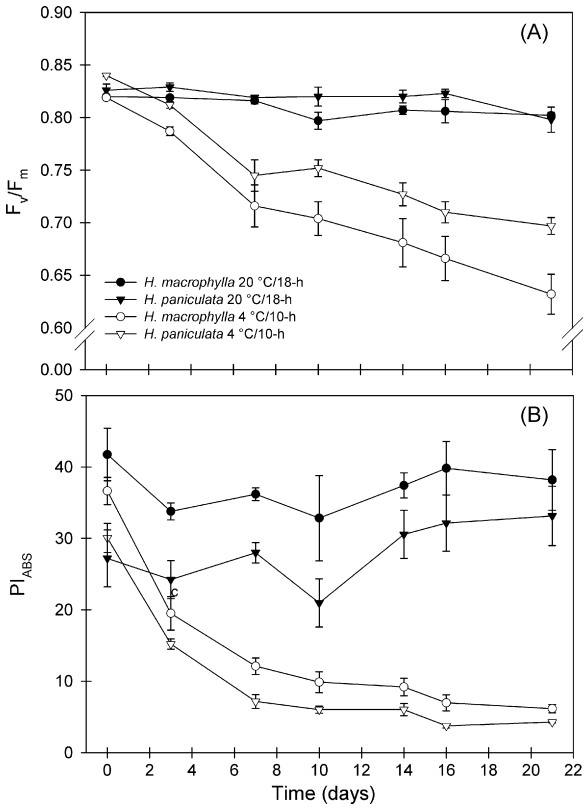

Fig. 3. Changes in (A) maximum photochemical efficiency of PSII in dark-adapted leaves (F_v/F_m) and (B) the performance index (PI_{ABS}) of *H. macrophylla* and *H. paniculata* exposed to 20 °C/18-h photoperiod or 4 °C/10-h photoperiod for 21 days. Values are mean ± S.E. of three to five replicates.

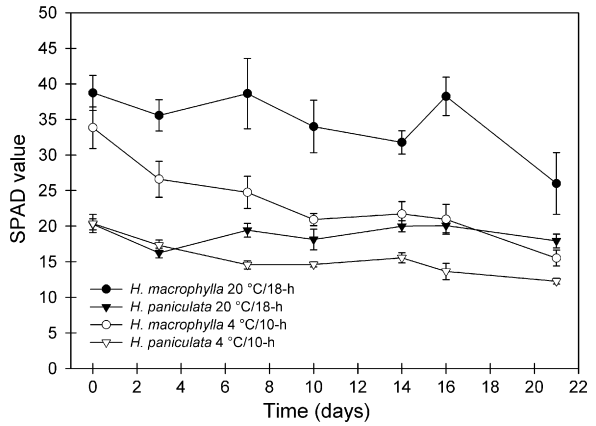

Fig. 4. Changes in chlorophyll meter readings (SPAD values) over time in leaves of *H. macrophylla* and *H. paniculata* subjected to 20 °C/18-h photoperiod or 4 °C/10-h photoperiod. Values are mean ± S.E. of three to five replicates.

did not indicate any species-specific differences in the effect of SD and LT on the photosynthetic apparatus. Separation of the multi-parametric PI_{ABS} into its partial responses supported the impression from the O–J–I–P transients. Hence, a decrease in the electron transport capacity ($\psi_o/(1-\psi_o)$), i.e. the probability to go from the reduced version of the first quinone electron acceptor in PSII (Q_A^-) to plastoquinone (PQ), which is sensitive to F_J and hence V_J was most pronounced at day 3, and greatest alterations in the efficiency of primary photochemistry ($\varphi_{Po}/(1-\varphi_{Po})$, related to the fluorescence extrema F_0 and F_m, appeared a day 7. The density of operative photosystems (reaction centres per chlorophyll, RC/ABS) was relatively unaffected by chilling stress (data not shown).

Values of SPAD were significantly affected by three double interactions (Table 2). Irrespective of treatment the chlorophyll content (SPAD units) of leaves was higher in *H. macrophylla* than in *H. paniculata* (Fig. 4). In control plants the leaf chlorophyll contents varied non-significantly between 26 and 39 units in *H. macrophylla* and between 16 and 20 units in *H. paniculata.* SD and LT resulted in a significant time-related loss of leaf chlorophyll in both species, but the chlorophyll loss was more pronounced in leaves of *H. macrophylla* than in leaves of *H. paniculata*, as indicated by the treatment × species interaction.

3.3. Carbohydrate concentrations

The leaf sucrose concentration was at all times higher in *H. paniculata* than in *H. macrophylla* (Fig. 5A, Table 2). Eleven days of SD and LT treatment increased the concentration significantly in both species, and at day 22 the concentration was approximately three times higher in SD- and LT-treated plants than in control plants of both species. In stems of control plants the concentration of sucrose was similar in the two species, but LT and SD induced a significant accumulation of sucrose in stems of *H. paniculata*, whereas in *H. macrophylla* only a smaller, non-significant increase was observed (Fig. 5B). As alterations in concentrations of glucose and fructose were similar within species and tissue types only concentrations of hexose (glucose + fructose) are shown. The leaf concentration of hexose was significantly increased by SD + LT treatment in both species, and by the interaction between treatment and time (Fig. 5C, Table 2). In *H. macrophylla* the stem concentration of hexose was rather high and the same level was found in control and SD + LT-treated plants (Fig. 5D, Table 2). In *H. paniculata* the hexose concentration of control stems was much lower than in *H. macrophylla*, but SD and LT induced a significant increase, which differed significantly from the concentration of control stems at both days 11 and 22.

3.4. Xylem sap [ABA]

The ABA concentration in xylem sap from the root system was significantly higher in SD + LT-treated plants than in control plants and it was significantly higher in *H. macrophylla* than in *H. paniculata* (Fig. 6A, Table 2). $[ABA]_{xylem}$ in control and stressed plants were on average 243 and 304 pmol mL^{-1}, respectively, while the average concentrations in *H. paniculata* and *H. macrophylla* were 237 and 309 pmol mL^{-1}. Although treatment duration did not significantly affect $[ABA]_{xylem}$ it clearly tended to increase transiently in both species (Fig. 6A). The concentration increased until the fourth day in *H. paniculata* and until the 8 day in *H. macrophylla*, thereafter it declined to levels somewhat higher than observed initially. Relative values for $[ABA]_{xylem}$ were calculated as the ratio between average $[ABA]_{xylem}$ of SD- and LT-treated plants to the average $[ABA]_{xylem}$ of control plants, which eliminates the species-specific difference in $[ABA]_{xylem}$ between *H. macrophylla* and *H. paniculata*. The relative values strengthens the suggestion concerning a transient increase (Fig. 6B), and indicate that SD and LT induced alterations in ABA levels of similar magnitude in the two species. However, the ABA peak level was observed earlier in *H. paniculata* than in *H. macrophylla.* The high relative $[ABA]_{xylem}$ values measured on day 1 indicate that $[ABA]_{xylem}$ already started to increase at day 0.

3.5. Freezing tolerance

The LT_{50} values of control plants varied non-significantly between −4.9 and −5.2 °C (Table 4). On day 5 freezing tolerance of

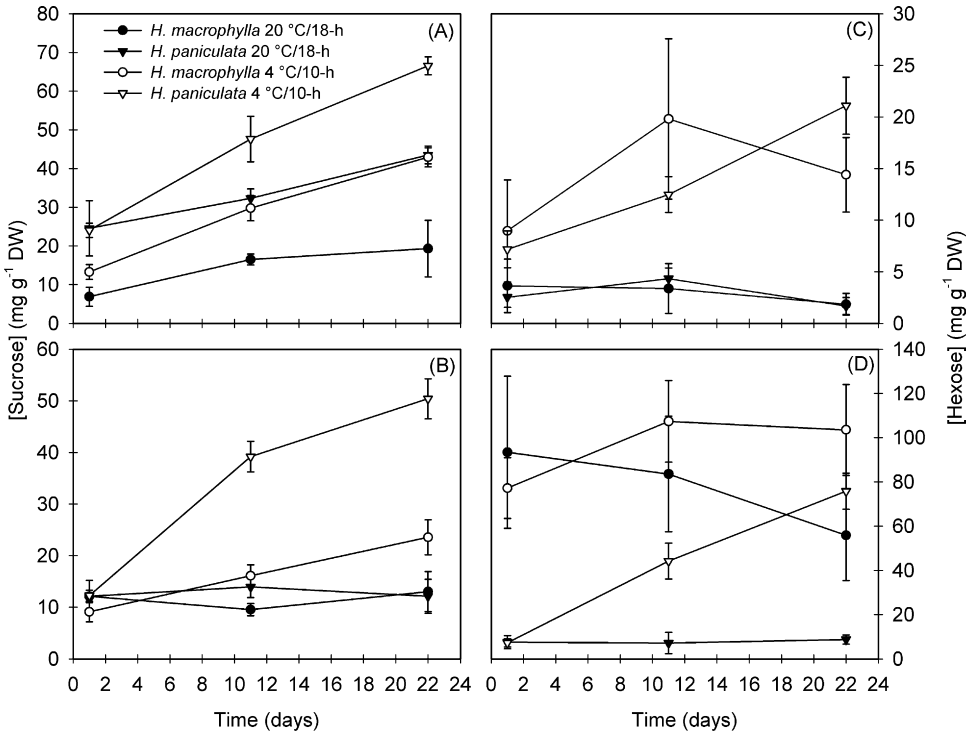

Fig. 5. Concentrations of sucrose ([Sucrose]) in (A) leaves and (B) stems and concentrations of hexose ([Hexose]) in (C) leaves and (D) stems of *H. macrophylla* and *H. paniculata* in the course of control temperatures and a long day photoperiod (20 °C/18-h) or in the course of chilling temperatures and a short day photoperiod (4 °C/10-h). Mean ± S.E., $n = 3–5$, except day 1, *H. macrophylla* leaves, 20 °C/18-h $n = 2$.

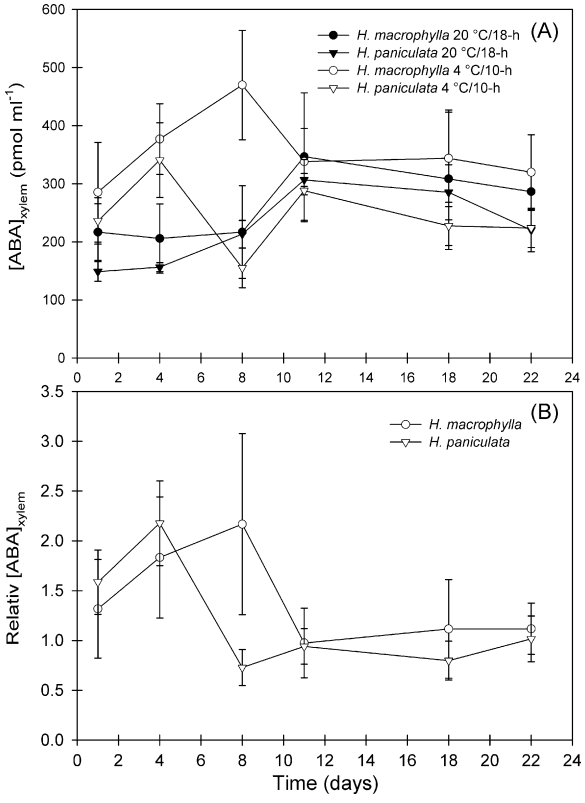

Fig. 6. (A) Changes in concentrations of ABA in xylem sap ($[ABA]_{xylem}$) and (B) the relative $[ABA]_{xylem}$ of *H. macrophylla* and *H. paniculata* during 22 days of short day and low temperature exposure (4 °C/10-h) in comparison with control plants (20 °C/18-h). Mean ± S.E., $n = 3–5$, except day 8, *H. paniculata*, 20 °C/18-h and day 11, *H. paniculata* and *H. macrophylla*, 20 °C/18-h $n = 2$. Data for relative $[ABA]_{xylem}$ was calculated as the ratio between average $[ABA]_{xylem}$ of short day and low temperature-treated plants to the average $[ABA]_{xylem}$ of control plants.

Table 4
Freezing tolerance assessed as LT_{50} values (temperatures representing 50% relative electrolyte leakage) of stems of *H. macrophylla* and *H. paniculata* during 25 days of exposure to 4 °C and a 10-h photoperiod in comparison with plants kept at control conditions (20 °C, 18-h)

Time (days)	LT_{50} (°C)	
	20 °C, 18-h	4 °C, 10-h
−2	-5.2 ± 0.1^{b}	-5.0 ± 0.1^{b}
5	-4.9 ± 0.1^{ab}	-4.6 ± 0.1^{a}
12	-5.2 ± 0.1^{b}	-5.1 ± 0.1^{b}
18	-5.2 ± 0.1^{b}	-5.3 ± 0.1^{b}
25	-5.2 ± 0.1^{b}	-5.3 ± 0.1^{b}

LT_{50} (mean ± S.E. (°C)) are shown for six (control) or 10 (short day and low temperature treated) plants. Different letters within the table indicate significant differences between LT_{50} values.

stems of SD + LT-treated plants was slightly, but significantly, less than during the rest of the treatment period. However, 25 days of an environmentally controlled, cold-acclimation regime did not increase stem freezing tolerance of either *H. macrophylla* or *H. paniculata* significantly, LT_{50} values of SD + LT-treated plants being on average −5.1 °C throughout the experiment.

4. Discussion

Results herein demonstrate that the level of stem hardiness in the non-acclimated state is similar in the two *Hydrangea* species, indicating that they are equally susceptible to cold spells in the growing season. They additionally demonstrate that an environmentally controlled, cold-acclimation regime (25 days exposure to a constant 4 °C and a 10-h photoperiod) is not sufficient to induce a significant increase in stem freezing tolerance in neither *H. macrophylla* nor *H. paniculata*, suggesting that *Hydrangea* may require temperatures lower than 4 °C to cold acclimate. Since SD and/or LT (4–5 °C) treatment has been shown to be sufficient to

increase freezing tolerance in many woody perennials [5,10,31,32], it was unexpected that *Hydrangea* apparently may require temperatures lower than 4 °C to start cold acclimation. However, the suggestion is supported by recent findings obtained in a field experiment running from September to May the following year, where a significant increase in stem cold hardiness of the same two cultivars was not observed until after a time period where several incidents of temperatures below 5 °C and two episodes of sub-zero temperatures occurred [33]. The results are additionally partly in line with data obtained by Adkins et al. [21], who suggested that sub-zero temperatures is an important hardiness-promoting factor in *H. macrophylla*. In ten different *H. macrophylla* varieties grown under natural cold acclimating conditions, they observed that stem cold hardiness varied between −3 and (−6) °C on 3 November. Approximately one month later, which included two non-consecutive weeks of freezing temperatures, cold hardiness in all cultivars had increased by at least −9 °C. If development of cold hardiness necessitates temperatures below 4 °C or even sub-zero temperatures in *H. macrophylla* and *H. paniculata* both species appear very susceptible to freezing injuries in areas with sudden temperature drops resulting in freezing events with temperatures below their constitutive hardiness level. It additionally indicates why *H. macrophylla* is considered to acclimate late. Different woody perennials acclimate differentially within a given temperature range [1], and although lack of increased stem freezing tolerance was unexpected, more plants have rather low threshold temperatures, above which no significant hardening occurs. In *Weigela* a range of cultivars acclimate late, with substantial hardening taking place concurrent with the minimum air temperature dropping to below ca. 5 °C on several occasions ([34], compare Table 1, Fig. 1). Likewise, in two populations of *Leptospermum scoparium* the apparent threshold temperature for the onset of frost hardening was about 6 °C [35].

As the conditions used in the present study did not induce cold acclimation, the observed responses cannot be directly related to changes in cold hardiness. However, the results help understanding how *Hydrangea* copes with chilling stress. The greater decrease in F_v/F_m observed in *H. macrophylla* than in *H. paniculata* indicates greater chilling sensitivity of leaves of the former than the latter species. In different genotypes of soybean the PI_{ABS} was a much more sensitive parameter for distinguishing differences in dark chilling response than F_v/F_m [24,25]. However, although the PI_{ABS} was also a more sensitive parameter than F_v/F_m in *Hydrangea*, and hence useful in early detection of chilling stress, it did not reveal any differences in SD and LT tolerance of leaves between the two species, indicating that in *Hydrangea* the PI_{ABS} may not necessarily be more suitable than F_v/F_m in distinguishing differences in chilling sensitivity. The J step of the O–J–I–P transient is believed to reflect light-driven accumulation of the reduced version of Q_A^- with the second quinone electron acceptor in PSII (Q_B) being oxidised. The increased fluorescence intensity at the J step observed primarily at day 3, but especially in *H. paniculata* also on subsequent days, may therefore reflect hindered electron transport downstream Q_A^-.The I-P phase has been suggested to reflect fluorescence quenching due to an oxidised plastoquinone pool. However, recent results by Tóth et al. [36] indicate that the F_m level is independent of the redox state of the plastoquinone pool, and the cause of the suppressed thermal phase of the Chl *a* fluorescence rise is therefore unknown [13]. The greater chilling tolerance of leaves of *H. paniculata* than of leaves of *H. macrophylla* may be advantageous in terms of cold hardiness of the whole plants. Lennartsson and Ögren [4] suggested that even deciduous trees may benefit from maintaining leaves as long as possible during autumn to allow continued photosynthesis, which may be important in building up reserves needed for cold acclimation. The stronger chlorophyll loss in leaves of *H. macrophylla* than in leaves of *H. paniculata* supports the assumption, that leaf senescence was accelerated in *H. macrophylla* compared to *H. paniculata*.

The present results support the supposition that ABA is involved in plant stress signalling in woody plants, including SD and/or LT responses in *Hydrangea*. The observed xylem sap ABA concentrations were within the range reported for field grown sugar maple until primo November, when the air and soil temperature was still above the freezing point [37,38]. In common with results obtained by Wan et al. [39] the present results indicate that low non-freezing air and/or soil temperatures increases ABA biosynthesis in the roots of woody plants and/or stimulates transport from the roots of either root-originated ABA or ABA produced elsewhere and transported to the roots [40]. In SD and/or LT-treated plants endogenous ABA has frequently been studied as a potential mediator for development of cold hardiness [5,10,37], but ABA from the root system has also been suggested to be implicated in the response of plants to low soil temperatures, either as a direct temperature effect or secondarily as a water-stress response induced by low soil temperature [39,41]. As the observed alterations in $[ABA]_{xylem}$ were not related to cold acclimation in the present study it was possibly a stress-response to low soil temperature. The soil temperature was not monitored, but it is anticipated that it was similar to the air temperature, as plants were kept in 3.5-L pots, limiting the volume of insulating soil. The ABA alterations observed in the current study resemble the patterns observed in the shoot apex and in leaves of LT-treated silver birch and sea buckthorn seedlings, respectively [5,6]. However, in both silver birch and sea buckthorn seedlings SD and LT separately or in combination resulted in increased hardiness, suggesting that ABA was implicated in the cold acclimation process. It is therefore possible that the alterations in aerial tissue concentrations of ABA in silver birch and sea buckthorn seedlings and the $[ABA]_{xylem}$ changes observed in the present study were involved in different processes and hence are not directly comparable. In aerial parts of herbaceous annual plants and over-wintering woody plants ABA concentrations have been shown to be more responsive to SD and/or LT conditions in the most chilling or freezing tolerant geno- or ecotypes [5,6,9]. In the present study the relative ABA peak level was similar in SD + LT-treated plants of the two species, but *H. paniculata*, which displayed greater chilling tolerance of leaves, was more responsive to SD and LT, resulting in an earlier ABA increase than observed in *H. macrophylla*.

Sucrose was the most abundant of the investigated soluble carbohydrates in leaves of both *Hydrangea* species, and sucrose also displayed the greatest increase during SD and LT treatment. Leaf concentrations of hexose increased slightly in SD + LT-treated plants, but were at all times higher in stressed than in control plants, which may be due to the fact, that the first destructive harvest took place on day 1, where the leaf hexose concentration may already have increased in response to SD and LT. Glucose, fructose and sucrose have previously been shown to accumulate in deciduous leaves of poplar exposed to chilling stress [42]. As growth was depressed in SD + LT-treated plants of both species a reduced demand for photosynthesis products may partially account for the increased sucrose concentrations, although photosynthetic yield was somewhat reduced. The magnitude of the increase in leaf sucrose was similar in the two species, but the initial sucrose concentration and hence the increased concentration in leaves of SD + LT-treated plants was significantly higher in *H. paniculata* than in *H. macrophylla*, which may be beneficial in terms of synthesis of stress-responsive compounds and supply of metabolic needs upon return to more favourable conditions [16].

H. paniculata additionally accumulated sucrose and hexose in stems in response to SD + LT, whereas in *H. macrophylla* only a small increase in the stem sucrose concentration was observed at day 22. Accumulation of soluble carbohydrates in over-wintering organs in response to SD and/or LT has often been correlated with increased hardiness, although not all studies have found a direct relationship between sugar concentrations and cold hardiness [43,44]. In the present study increased concentrations of sugars in stems of *H. paniculata* did not play a direct role in cold hardiness but may be a consequence of an altered balance between photosynthesis and growth and hence export of supplementary photosynthates.

In conclusion, SD and LT induce various stress-coping mechanisms in *Hydrangea*, including growth cessation, perturbation of PSII, loss of chlorophyll and accumulation of sugars in leaves and stems. Leaves of *H. macrophylla* are more sensitive to chilling and show accelerated senescence compared to leaves of *H. paniculata*. *H. macrophylla* additionally accumulates less protective and immediate convertible sugars in leaves and stems than *H. paniculata* in response to SD + LT. Probably as a response to low soil temperature both species display a transient increase in $[ABA]_{x\text{-}ylem}$, with *H. paniculata* responding faster than *H. macrophylla*, suggesting faster acquisition of tolerance to low soil temperature. Cold hardiness in the non-acclimated state is similar in *H. macrophylla* and *H. paniculata* and development of cold hardiness probably requires temperatures below ca. 4 °C in both species, rendering them equally sensitive to frost episodes in the growing season and to sudden temperature drops in the autumn.

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Changes in carbohydrates, ABA and bark proteins during seasonal cold acclimation and deacclimation in *Hydrangea* species differing in cold hardiness

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Cold injury is frequently seen in the commercially important shrub *Hydrangea macrophylla* but not in *Hydrangea paniculata*. Cold acclimation and deacclimation and associated physiological adaptations were investigated from late September 2006 to early May 2007 in stems of field-grown *H. macrophylla ssp. macrophylla* (Thunb.) Ser. cv. Blaumeise and *H. paniculata* Sieb. cv. Kyushu. Acclimation and deacclimation appeared approximately synchronized in the two species, but they differed significantly in levels of mid-winter cold hardiness, rates of acclimation and deacclimation and physiological traits conferring tolerance to freezing conditions. Accumulation patterns of sucrose and raffinose in stems paralleled fluctuations in cold hardiness in both species, but *H. macrophylla* additionally accumulated glucose and fructose during winter, indicating species-specific differences in carbohydrate metabolism. Protein profiles differed between *H. macrophylla* and *H. paniculata*, but distinct seasonal patterns associated with winter acclimation were observed in both species. In *H. paniculata* concurrent increases in xylem sap abscisic acid (ABA) concentrations ($[ABA]_{xylem}$) and freezing tolerance suggests an involvement of ABA in cold acclimation. In contrast, ABA from the root system was seemingly not involved in cold acclimation in *H. macrophylla*, suggesting that species-specific differences in cold hardiness may be related to differences in $[ABA]_{xylem}$. In both species a significant increase in stem freezing tolerance appeared long after growth ceased, suggesting that cold acclimation is more regulated by temperature than by photoperiod.

Introduction

Geographical distribution and cultivation of horticultural crops in temperate climates are strongly dependent on their freezing tolerance. Increased freezing tolerance (beyond a 'constitutive' level) can be acquired by the process of cold acclimation, which involves physiological and biochemical changes where plants become increasingly tolerant to subzero temperatures (Guy 2003, Li et al. 2004, Weiser 1970). Cold acclimation is a seasonal process, with freezing tolerance increasing during the autumn, reaching its maximum mid-winter and declining in the spring. Susceptibility of plants to frost injury may therefore not only be ascribed to insufficient maximum freezing tolerance, but also to the timing and rates of acclimation and deacclimation (Suojala and Lindén 1997). Parallel to cold acclimation temperate

Abbreviations – ABA, abscisic acid; DW, dry weight; FW, fresh weight; LT, low temperature; LT_{50}, temperature representing 50% REL; REL, relative electrolyte leakage; SD, short photoperiod.

woody plants form terminal buds and develop dormancy (Rohde and Bhalerao 2007). In numerous horticultural crops, knowledge about freezing tolerance and the dynamics of both cold acclimation and deacclimation and the underlying physiological and biochemical processes is therefore of both theoretical and commercial importance.

Levels of freezing tolerance and associated adaptations vary between species, cultivars, ecotypes etc., demonstrating genetic variability for cold hardiness and genetic adaptation to the local climate (Arora et al. 1996, Kalberer et al. 2007, Lim et al. 1999, Reyes-Días et al. 2005, Rowland et al. 2005, Schrader and Graves 2003). Key physiological responses during cold acclimation include decreased tissue water content and accumulation of compatible compounds. Compatible compounds, such as soluble carbohydrates, amino acids and certain proteins, may function in protecting cell structures from freeze-induced dehydration and/or in freezing point depression (Li et al. 2004, Xin and Browse 2000). The close involvement of soluble carbohydrates in freezing tolerance is well established (Uemura et al. 2003, Wanner and Junttila 1999), and in many woody plants cold hardiness has been found to be correlated to the concentration of soluble carbohydrates (Lennartsson and Ögren 2003, Palonen et al. 2000). Sucrose is probably the most studied soluble carbohydrate in relation to cold tolerance, but also the raffinose family of oligosaccharides (raffinose and stachyose) has been associated with season-long freezing tolerance in woody perennials (Cox and Stushnoff 2001, Imanishi et al. 1998, Palonen et al. 2000). In addition to altered carbohydrate metabolism, differences in cold hardiness have been associated with both qualitative and quantitative changes in proteins. Some of these seasonally fluctuating proteins have been characterized, e.g. dehydrins, which are induced by conditions that cause cellular dehydration (Arora and Wisniewski 1994, Close 1996, Harris et al. 2006, Karlson et al. 2003, Marian et al. 2004, Muthalif and Rowland 1994, Renaut et al. 2005). In some woody perennials, protein expression profiles among related species or genotypes are associated with genotype-specific freezing tolerance (Arora et al. 1992, Lim et al. 1999).

Changes in water status and protein levels and patterns in cold acclimating plants have been suggested to be induced by the phytohormone abscisic acid (ABA) (Rinne et al. 1998, Wisniewski et al. 2006). Additionally, an increasing amount of evidence links ABA and soluble carbohydrates via their response pathways (Gibson 2003, Rook et al. 2006), although there have been no attempts to investigate such interactions in cold acclimating woody plants. In field-grown woody perennials the xylem sap ABA concentration has been shown to be markedly elevated during winter. Increased xylem sap ABA concentrations have been suggested to be involved in controlling dormancy (Alvim et al. 1976, Davison and Young 1974) and cold acclimation (Bertrand et al. 1997, 1999). ABA also increases in aerial parts of woody plants in response to short days (SDs) and/or low temperatures (LTs). However, in aerial plant parts endogenous ABA concentrations often display a short-term (days–weeks) transient increase, preceding development of freezing tolerance (Li et al. 2002, Rinne et al. 1998, Welling et al. 2002). As a signal transducer ABA has been suggested to be important for controlling the rate and degree of cold acclimation, i.e. earlier cold acclimation and/or higher freezing tolerance may be related to faster or more prominent alterations in ABA levels in related ecotypes (Li et al. 2005a, 2005b).

Previous studies have shown that maximum freezing tolerance of *Hydrangea* L., a popular and widely used woody ornamental, varies considerably among the two species *H. macrophylla* and *H. paniculata* (Adkins et al. 2003, Suojala and Lindén 1997), but the physiological reasons for this difference in cold hardiness are unknown. Not only is *H. macrophylla* less freezing tolerant than *H. paniculata*, the consequences of freezing injuries in terms of quality and ornamental value are of horticultural importance. Flower buds of most *H. macrophylla* varieties are formed during the autumn and overwinter on dormant stems, flowering will therefore only occur the following year if terminal and/or lateral flower buds are present and undamaged. In contrast, on *H. paniculata* flowers usually originate from current season's shoots.

The present study was conducted to investigate (1) the association between seasonal changes in selected physiological parameters and cold hardiness in *H. macrophylla* and *H. paniculata* and (2) to determine differences in cold hardiness during autumn acclimation and spring deacclimation. Particularly, an attempt was made to relate alterations in xylem sap ABA concentrations, stem water contents and concentrations of starch and soluble carbohydrates to seasonal changes in stem freezing tolerance in the two species. Additionally, seasonal qualitative protein changes were determined to provide a basis for further, more detailed protein analytic studies.

Materials and methods

Location, climate and plant material

The experiment was carried out using 2-year-old vegetatively propagated and commercially produced *H. macrophylla ssp. macrophylla* (Thunb.) Ser. cv. Blaumeise and *Hydrangea paniculata* Sieb. cv. Kyushu plants grown in 3.5-l pots containing sphagnum peat. During the

experimental period (from September 27, 2006 to May 10, 2007), the potted plants were maintained outside at Research Centre Aarslev in Aarslev, Denmark (latitude 55° 01′N, altitude 9 m) with pots buried in the soil to avoid root frost injuries. Local air temperature and day length data were obtained from the research centers climate station, which is operated by the Danish Meteorological Institute. The soil temperature was monitored with Tinytalk temperature loggers (Gemini Data Loggers, Chichester, UK) buried between pots at a depth of ca. two thirds of the pot height. The winter of 2006 turned out to be relatively warm, and the minimum air and soil temperatures did not reach −10°C at any time (Fig. 1). In the months of September, October, November and December the air temperature only dropped below the freezing point on two occasions. The lowest air temperature of −7.3°C occurred at the end of January. From January to April, inclusive, 20 days had temperatures below zero. The soil temperature was around the freezing point in late January and mid-February but it did not drop below 0°C during the experimental period. At the beginning of the experiment the day length was 12.5 h, this decreased to a minimum of ca. 7 h around January, and again it increased to above 15 h in May.

The pots were surrounded by sand so they could be removed from the soil during the winter months and brought to the laboratory to collect xylem sap and prepare samples for freeze test. Measurements and sampling of current year shoots were carried out approximately once every month from September 2006 to May 2007. For measurements of elongation growth and collection of xylem sap for determination of ABA concentrations, the last measurement/sampling date was March 28, 2007.

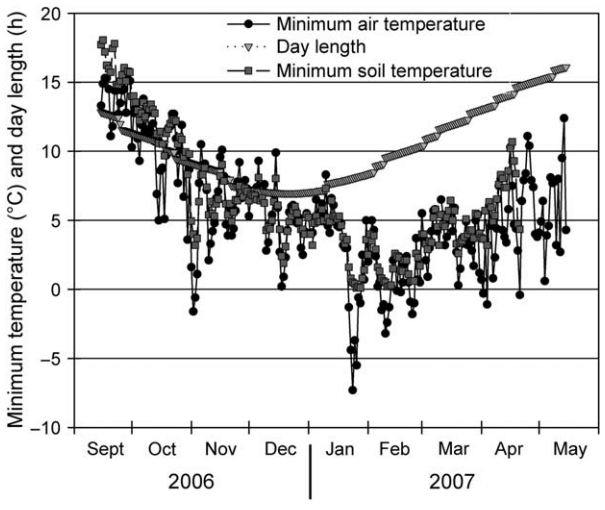

Fig. 1. Day length and minimum daily air and soil temperatures at the experimental site from September 2006 through May 2007.

Samples were randomly collected from six plants per species.

Elongation growth and tissue water contents

Elongation growth was determined with a ruler measuring the distance between the shoot tip and a reference mark made between the tip and the first node. Bud set was determined by examining the shoot apex.

Fresh weight (FW) and dry weight (DW) of leaves and stems were recorded before and after drying to constant weight at 80°C. The tissue water contents were then calculated.

Determination of freezing tolerance

Freezing tolerance was determined on a seven test temperature series, by measuring the extent of freezing injury by a slightly modified version of an electrolyte leakage method previously described for other woody plants (Brønnum 2005). The selected temperatures varied depending on the month and the result of the preceding test. One (*H. macrophylla*) or two (*H. paniculata*) 3-cm-long pieces of internodal stem tissue were rinsed under cold running tap water for 15 s and then under cold running demineralized water for 15 s. After rinsing the samples were placed in 70-ml test tubes and incubated in a controlled freezer. Few drops of demineralized water were added to each sample to initiate ice formation. The samples were cooled at a rate of maximum 5°C/h to 0°C and subsequently at 2°C/h until the selected temperature was reached. The selected temperature was maintained for 2 h, thereafter the samples were withdrawn and thawed at 4°C. Ions were extracted with 35 ml of demineralized water for 20 h at room temperature and the electrical conductivity measured (EC_{frozen}) using a ION570 ISE-meter with temperature-corrected display (Radiometer, Copenhagen, Denmark). After determination of the EC the samples were autoclaved for 1 h to allow maximum leakage of ions. After autoclaving the samples were allowed to cool to room temperature and the EC was measured again ($EC_{autoclave}$). The EC of demineralized water (EC_{water}) was measured to give the zero level of EC. Relative electrolyte leakage (REL) was calculated as $REL = (EC_{frozen} - EC_{water}) \times 100/(EC_{autoclave} - EC_{water})$.

Protein extraction and analysis

Bark proteins from lyophilized powdered bark tissues were first extracted using a modified version of a borate buffer-based extraction protocol previously successfully used for other woody plants (Arora and Wisniewski 1994,

Lim et al. 1999). However, this method consistently resulted in poor quality gels, especially for *H. macrophylla*, whereby visual band intensities reflected exceedingly lower amount of protein than what was actually loaded on the gel based on protein estimation. Hence, another extraction protocol, a phenol-based, was employed for this study (see Discussion for detailed rationale). Approximately 0.35 g of polyvinylpolypyrrolidone was suspended in 5 ml of extraction buffer [0.5 *M* Tris, 50 m*M* ethylenediaminetetraacetic acid, 100 m*M* KCl, 0.7 *M* sucrose, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 100 μ*M* N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), pH 7.5], 0.5 g bark tissue was added, vortexed and homogenized at 4°C on a shaker. After 10 min of homogenization the mixture was adjusted to 40 m*M* dithiotreitol and homogenized for another 20 min at 4°C. The mixture was centrifuged at 4°C for 30 min at 20 000 *g* and the supernatant was removed and combined with an equal volume of Tris-buffered phenol (pH 8.0). Resulting mixture was shaken for 30 min at 4°C and centrifuged at 4°C for 30 min at 10 000 *g*. The upper phenol phase was carefully recovered and combined with a five-fold volume of cold (−20°C) 0.1 *M* ammonium acetate in methanol and stored at −20°C overnight. The following day proteins were precipitated by centrifugation (10 000 *g* for 30 min at 4°C) and washed twice with 5 ml of cold (−20°C) 0.1 *M* ammonium acetate in methanol and once in 5 ml cold (−20°C) acetone. Following each wash the protein precipitate was collected by centrifugation (20 000 *g* for 20 min at 4°C). After drying the pellet was resuspended in 0.25 *M* Tris–HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8, boiled for 5 min and cooled to room temperature.

Protein concentrations were measured using the method of Esen (1978). Proteins were separated by discontinuous SDS–polyacrylamide gel electrophoresis (SDS-PAGE) performed with a Mini-Protean II electrophoresis unit (Bio-Rad, Hercules, CA) using 4% stacking gel and 12.5% separating gel. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 and destained with 25% methanol. As equal loadings among, but not within, species revealed (visually) different amounts of protein in various gel lanes, a total of 15 and 8 μg of protein from samples of *H. macrophylla* and *H. paniculata*, respectively, were loaded in each lane. Differences between measured protein concentrations and amounts of protein revealed by stained gel profiles indicate that either the protein estimation method perhaps over- or under estimated the protein concentration in *H. paniculata* and *H. macrophylla*, respectively, or about half the protein loaded for *H. macrophylla* did not enter the gels during separation. Because Esen's method has been well tested for its estimation accuracy in previous studies (Lim et al. 1999, Marian et al. 2004), the latter explanation seems more likely (also see Discussion).

Collection of xylem sap and determination of xylem sap [ABA]

To collect xylem sap potted plants were transferred to a Scholander-type pressure chamber and decapitated approximately 5 cm above the soil surface. With the stem stump protruding outside the chamber, pressure was applied until the root water potential (Ψr) was equalized. After determination of Ψr the cut surface was cleaned with pure water, dried with blotting paper and the root system was gradually pressurized and then maintained at ca. −0.5 MPa. Xylem sap was collected for 5–20 min and thereafter stored at −80°C, except during a breakdown of the −80°C freezer where samples were kept at −20°C, until further analysis. The ABA concentration in xylem sap was determined without purification by an enzyme-linked immunosorbent assay using a monoclonal antibody for ABA (AFRC MAC 252) according to Asch et al. (2001). To verify that the xylem sap did not contain compounds which reacted with the antibody other than the antigen a cross reaction test was performed for both *Hydrangea* species.

Soluble carbohydrate analysis

Approximately 3 g FW of current year stems were harvested and frozen in liquid nitrogen for determination of carbohydrates. Concentrations of glucose, fructose, sucrose and raffinose were determined using high-performance liquid chromatography (Hewlett Packard 1047A, Waldbronn, Germany) as described by Liu et al. (2004). Starch was determined in the remaining pellets after extraction of soluble carbohydrates as described by Pagter et al. (2008b).

Data analysis

Data of tissue water contents, concentrations of carbohydrates in stems and concentrations of ABA in xylem sap were analyzed using two-way analysis of variance (ANOVA) and Type III sum of squares (PROC GLM of SAS, SAS Institute, Cary, NC). Main effects were considered to be species and time (treatment duration), as well as their interaction. Heterogeneities of variance within species at each time separately were tested using Bartlett's Test. When necessary data were log-transformed to ensure homogeneity of variance, but for clarity all data are presented as untransformed. Differences between individual means were identified using Tukey's Studentized

Range Test at the 5% significance level. A non-parametric Kruskall–Wallis test was also used to test differences in stem water contents and concentrations of starch, raffinose, glucose and fructose between species and treatment durations. This test was done to supplement the result from the two-way ANOVA, as the assumption concerning similar variance was not fulfilled. Two-way ANOVA and Kruskall–Wallis tests gave similar results when assessing effects of different species and treatment durations on stem water contents and concentrations of starch, raffinose and fructose and only the results of the ANOVA are described. When examining differences in concentrations of glucose the ANOVA indicated a significant effect of species, treatment duration and their interaction whereas the Kruskall–Wallis tests only indicated a significant effect of species. However, when glucose results were analyzed for each species separately both the parametric and non-parametric tests indicated a significant effect of treatment duration and hence the results of a one-way ANOVA for each species separately are described.

Freezing tolerance was estimated as LT_{50} values, the temperature representing 50% REL. At each time separately data for all six replicates per treatment were fitted by regression analysis (PROC NLIN) to the sigmoid function $REL = REL_{min} + (REL_{max} - REL_{min})/(1 + \exp(c \times (d - T)))$, where REL_{min} is the base line of REL, REL_{max} is the maximum REL, c is the slope of the function at the inflection point d and T is the treatment temperature. The temperature (d) at the inflection point was used as LT_{50} (Väinolä and Repo 1999). Differences between LT_{50} estimates were taken as significant if the 95% confidence intervals did not overlap. Correlation coefficients between concentrations of soluble carbohydrates and LT_{50} values and between number of hours where the mean temperature was <5°C and LT_{50} values were examined using Pearson's correlation coefficient (PROC CORR).

Results

Freezing tolerance

Freezing tolerance of stems differed significantly according to sampling date, but in different ways in the two species ($P < 0.05$, Fig. 2). In September and October freezing tolerance of stems was ca. −5°C in both species. Between October 25 and January 31 freezing tolerance increased in successively later sampling periods reaching a maximum of ca. −17°C and < −30°C in *H. macrophylla* and *H. paniculata*, respectively. At November 25 and between January 3 and February 28, stem freezing tolerance of *H. paniculata* could not be precisely evaluated, as the minimum freezing temperature used at November 25 was −20°C and between January 3 and February 28 the lowest temperature reached by the freezer was −30°C. From the end of February to mid-May freezing tolerance decreased to −4°C in *H. macrophylla* and to −8°C in *H. paniculata*. During acclimation, until maximum freezing tolerance was reached (September through January), LT_{50} values of *H. macrophylla* correlated well with the number of hours where the mean temperature was less than 5°C (Table 1). In *H. paniculata* the correlation was less strong. However, a cautious interpretation of the result of the correlation between number of hours with a mean temperature < 5°C and LT_{50} values of *H. paniculata* is required, as 'estimated' LT_{50} values of −20°C at November 25 and −30°C between January 3 and February 28 were used.

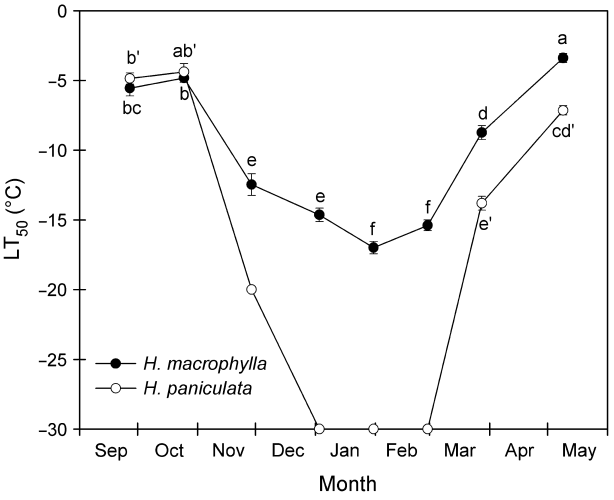

Fig. 2. Seasonal changes in cold hardiness estimated as temperatures representing 50% relative electrolyte leakage (LT_{50}) of stems of *Hydrangea macrophylla* and *Hydrangea paniculata*. LT_{50} [mean ± SE (°C)] are shown for six plants tested at seven temperatures. LT_{50} values of stems of *H. paniculata* were lower than −20°C at November 25 and lower than −30°C between January 3 and February 28 but could not be precisely determined, as the minimum test temperature used at November 25 was −20°C and between January 3 and February 28, the lowest temperature reached by the freezer was −30°C. Different letters indicate significant differences between species and sampling dates ($P < 0.05$). Differences in LT_{50} values of *H. paniculata* are indicated by marked letters for clarity. Rates of acclimation and deacclimation were calculated as Δfreezing tolerance/Δtime, where Δfreezing tolerance; minimum LT_{50} value − LT_{50} value in September (rate of acclimation) or May (rate of deacclimation) and Δtime; number of days in the corresponding time periods.

Elongation growth and tissue water contents

As the experimental plants had been kept outdoors since the previous winter, elongation growth had ceased when the experiment was started in late

Table 1. Correlation coefficients between stem cold hardiness estimated as temperatures representing 50% relative electrolyte leakage (LT_{50}) and concentrations of different soluble carbohydrates in stems of field-grown *Hydrangea macrophylla* and *Hydrangea paniculata* from September to May, and between stem cold hardiness and number of hours with a mean temperature < 5°C from start of the experiment and until maximum hardiness was reached (September through January). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. As LT_{50} values of *Hydrangea paniculata* could not be precisely evaluated at November 25 and between January 3 and February 28, estimated LT_{50} values of −20°C on November 25 and −30°C between January 3 and February 28 were used.

	Correlation coefficient	
	Hydrangea macrophylla	*Hydrangea paniculata*
Carbohydrate		
Raffinose	−0.80*	−0.89**
Sucrose	−0.96***	−0.90**
Glucose	−0.79*	0.69 ns
Fructose	−0.91**	0.21 ns
Hours with a mean temperature <5°C	−0.91*	−0.87*

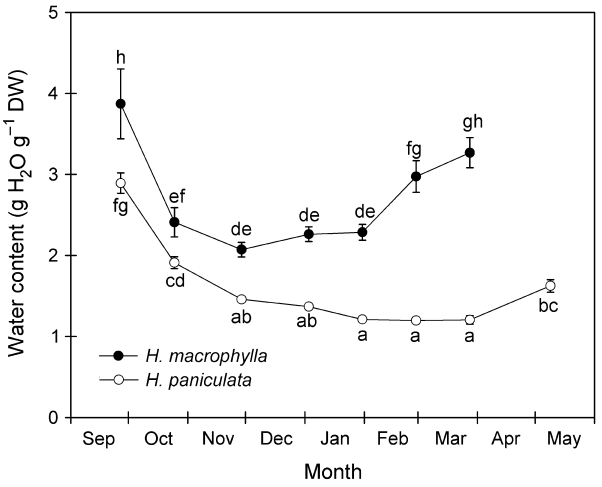

Fig. 3. Seasonal changes in stem water content of *Hydrangea macrophylla* and *Hydrangea paniculata*. By mistake the stem water content of *H. macrophylla* was not determined in May because of logistical reasons. Values are means ± SE of six replicates. Different letters indicate significant differences between species and sampling dates ($P < 0.05$).

September, indicating that in both *H. macrophylla* and *H. paniculata* the critical photoperiod for growth cessation is longer than ca. 12.5 h. Terminal buds were formed between October 23 and November 27, but between January 3 and January 29 most terminal and axillary buds of *H. macrophylla* were killed. The leaf water content was only determined on the first two sampling dates, as most leaves had abscised on subsequent sampling dates. No significant changes in the leaf water contents were observed from September to October, but the water content was significantly higher in leaves of *H. macrophylla* than in leaves of *H. paniculata* ($P < 0.001$), being on average 5.3 g g^{-1} DW and 3.9 g g^{-1} DW, respectively. As shown in Fig. 3 alterations in the stem water content differed between the two species ($P < 0.001$). During the autumn it decreased consecutively in both species, reaching minimum in late November. In February and March the stem water content of *H. macrophylla* increased to the level observed in September, whereas in *H. paniculata* increasing stem water contents were not observed until mid-May. Mistakenly the stem water content of *H. macrophylla* was not determined in May because of logistical reasons.

Protein patterns

The protein patterns in one dimensional SDS-PAGE showed seasonal changes and qualitative differences between the two species (Fig. 4). In both species a polypeptide with an estimated molecular mass of 60 kDa accumulated during the autumn and winter and decreased during spring, most notably in *H. macrophylla*. Another common protein at approximately 22 kDa accumulated to high levels in *H. paniculata* during November to March but showed no clear seasonality in *H. macrophylla*. In *H. macrophylla*, two proteins at ca. 43 and 18 kDa increased and then decreased corresponding to autumn/winter and spring. Although not present in March the 18-kDa protein reappeared in May. In *H. paniculata*, two almost similar sized proteins (ca. 33 and 34 kDa) and a 27-kDa protein also showed a seasonal pattern.

Compared to the results of the phenol-based extraction described above, borate buffer extraction and trichloroacetic acid (TCA)-precipitation of *H. macrophylla* samples resulted in low quality gels with severe background, faint protein bands and low resolution. In *H. paniculata*, however, the two extraction methods resulted in gels of similar quality (visual observation) and comparable protein patterns (data not shown).

Xylem sap [ABA]

The $[ABA]_{xylem}$ was significantly affected by sampling date but in different ways for the two species ($P < 0.001$). In *H. macrophylla* $[ABA]_{xylem}$ decreased from around 600 pmol ml^{-1} in September and October to a minimum level around 95 pmol ml^{-1} in late January and until the end of the experiment (Fig. 5). Conversely, in *H. paniculata* strong seasonal alternations in $[ABA]_{xylem}$ were observed, the concentration increased from ca. 640 pmol ml^{-1} on September 25 and October 25 to

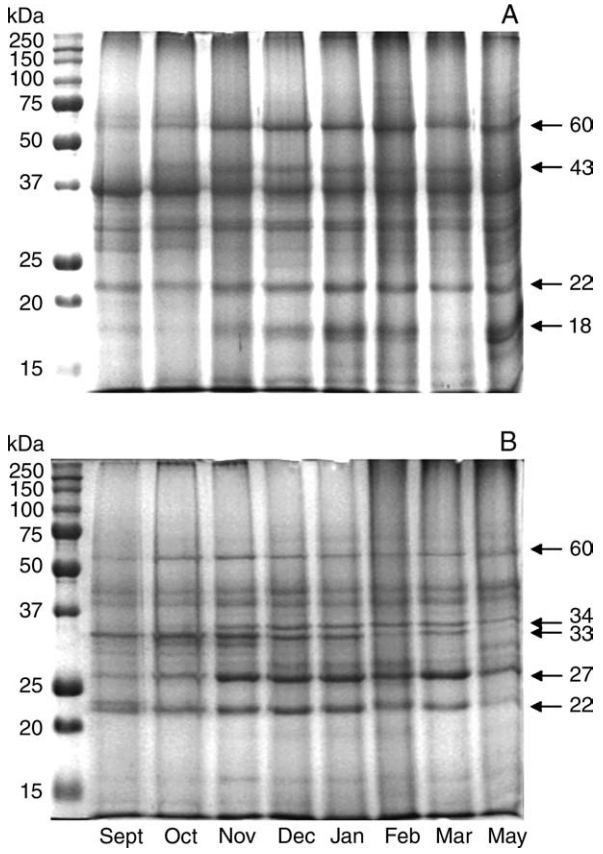

Fig. 4. Seasonal SDS-PAGE profiles of bark proteins of *Hydrangea macrophylla* (A) and *Hydrangea paniculata* (B). A total of 15 and 8 μg of protein from samples of *H. macrophylla* and *H. paniculata*, respectively, were loaded in each lane. Molecular masses of the markers are indicated to the left while those of bark proteins on the right with arrows.

a maximum of 5500 pmol ml^{-1} on January 31. While by March 28 the concentration had decreased to a level comparable to the concentrations measured in September and October.

Carbohydrate concentrations

The concentration of starch differed significantly between sampling dates but in slightly different ways in the two species ($P < 0.001$). From initiation of the experiment and until leaf fall (between the second and the third sampling time) the stem starch concentration increased in both species (Fig. 6A). Thereafter, concentrations of starch decreased to a winter minimum, which persisted until the end of February. At the time of budburst and growth of leaves in spring (end of April – beginning of May) starch was completely mobilized. The concentration of starch in stems was constantly higher in *H. macrophylla* than *H. paniculata*, on average 39% throughout the experiment.

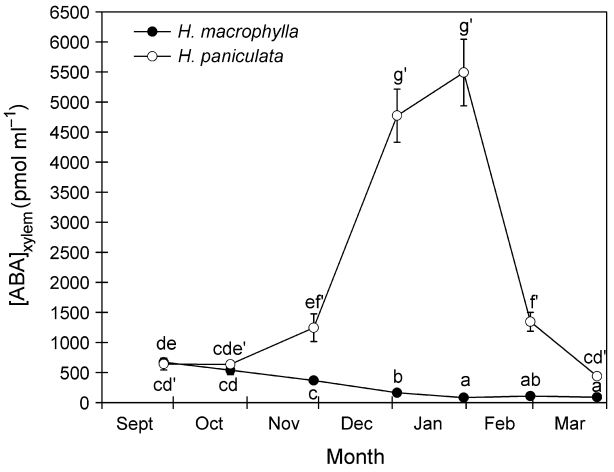

Fig. 5. Changes in xylem sap ABA concentrations ($[ABA]_{xylem}$) in *Hydrangea macrophylla* and *Hydrangea paniculata* from the end of September and until the end of March the following year. Mean ± SE, n = 6 except January 3, *H. paniculata* n = 4 and January 31, *H. macrophylla* and *H. paniculata* n = 5. Different letters indicate significant differences between species and sampling dates ($P < 0.05$). Differences in $[ABA]_{xylem}$ of *H. paniculata* are indicated by marked letters for clarity.

The concentrations of four different soluble carbohydrates, namely sucrose, glucose, fructose and raffinose, were analyzed in stem samples. Concentrations of soluble carbohydrates varied to different extents depending on the nature of the carbohydrate considered and the plant species ($P < 0.001$), but generally concentrations of all quantified soluble carbohydrates were considerably higher in *H. macrophylla* than in *H. paniculata*. In *H. paniculata* raffinose (Fig. 6B) and sucrose (Fig. 6C) were the most abundant soluble carbohydrates and they both displayed a seasonal trend. Their concentrations increased during cold acclimation in the autumn, reached a peak in winter, and decreased in the spring. Hence, both the raffinose and sucrose concentrations in *H. paniculata* presented high correlations with LT_{50} values (Table 1). On the contrary, concentrations of glucose (Fig. 6D) and fructose (Fig. 6E) were low and did not correlate with season or cold hardiness neither during acclimation nor deacclimation in *H. paniculata*. In *H. macrophylla* raffinose and sucrose also displayed a seasonal trend, with raffinose reaching a peak level earlier (early January) than sucrose (late January) and before maximum freezing tolerance was reached. Initially concentrations of glucose and fructose decreased, although not significantly, in stems of *H. macrophylla* but their levels started to increase along with the observed increase in stem freezing tolerance. From then on the concentrations of glucose and particularly fructose increased and decreased in parallel with seasonal alterations in stem freezing tolerance. In stems of

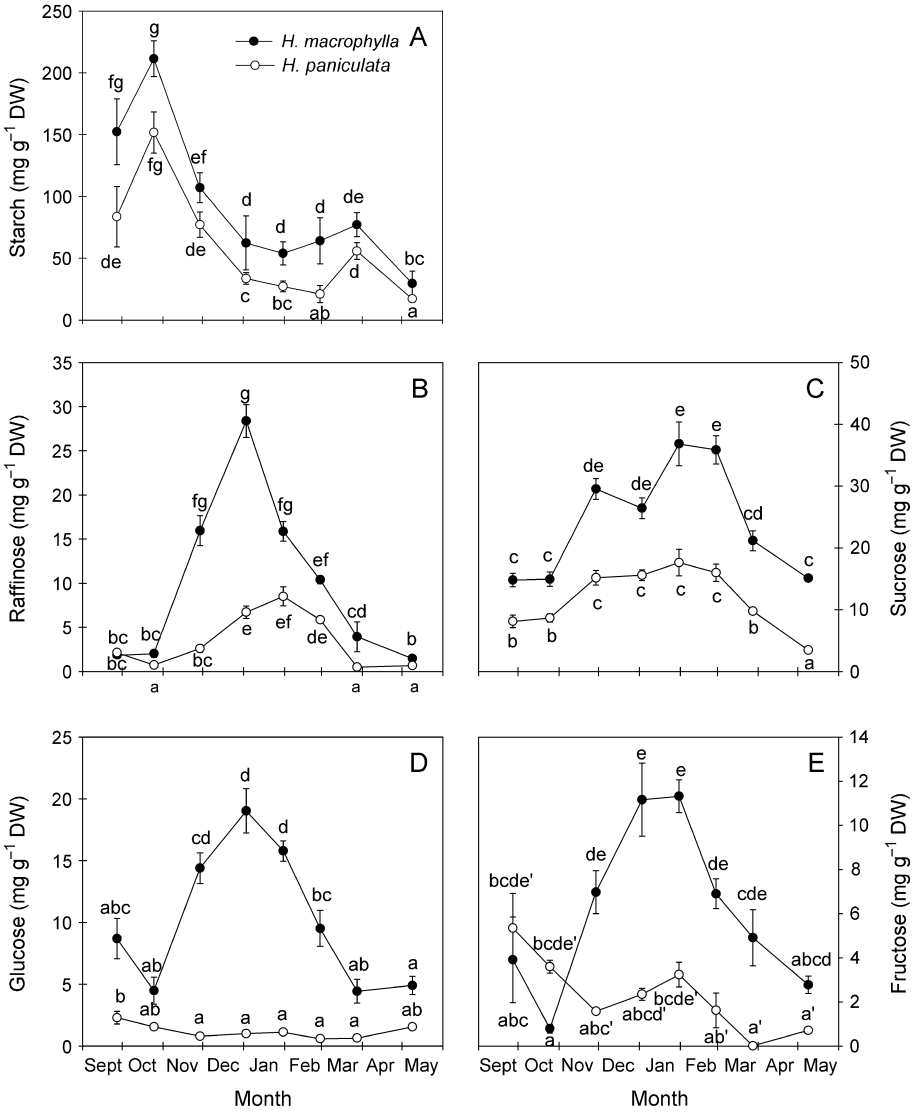

Fig. 6. Changes in concentrations of starch (A), raffinose (B), sucrose (C), glucose (D) and fructose (E) in stems of *Hydrangea macrophylla* and *Hydrangea paniculata* from the end of September and until mid-May the following year. Values are means ± SE of n = 5–6. In (A), (B), (C) and (E) different letters indicate significant differences between species and sampling dates ($P < 0.05$), whereas in (D) different letters indicate significant differences between sampling dates for each species separately ($P < 0.05$). In Fig. 6E differences in fructose concentrations in *H. paniculata* are indicated by marked letters for clarity.

H. macrophylla, all quantified soluble carbohydrates were correlated with LT_{50} values (Table 1).

Discussion

Hydrangea macrophylla and *H. paniculata* acclimated approximately simultaneously, but, as expected, *H. paniculata* developed considerably greater stem cold hardiness than *H. macrophylla*. Minimum LT_{50} values of −17°C and < −30°C (Fig. 2) observed in stems of *H. macrophylla* and *H. paniculata*, respectively, correspond well with previously reported lowest survival temperatures in ten different *H. macrophylla* varieties (Adkins et al. 2003) and LT_{50} values based on visual assessments in three different *H. paniculata* cv. Grandiflora clones (Suojala and Lindén 1997). *H. macrophylla* and *H. paniculata* also started to de-acclimate at approximately the same time, but although the rate of deacclimation was higher in *H. paniculata* than in *H. macrophylla*, −0.23 and −0.14°C per day respectively, the lower absolute freezing tolerance during deacclimation meant that stems of *H. macrophylla* were much more susceptible to spring

frost than stems of *H. paniculata*. Additionally, buds of *H. macrophylla* appeared to be even less freezing tolerant than stems, as most terminal and axillary buds of *H. macrophylla* were damaged or killed in January (visual observation), when the lowest air temperatures were recorded. Although plants were able to produce new shoots in spring from buds positioned near the soil surface, it reinforces the vulnerability of *H. macrophylla* compared with *H. paniculata*.

Our results suggest that induction of cold acclimation in *H. macrophylla* and *H. paniculata* is mainly regulated by temperature rather than by photoperiod, or the effect of photoperiod may be indirect. In late September growth had ceased in both *H. macrophylla* and *H. paniculata*, indicating that both species had perceived a SD signal before initiation of the experiment (Rohde and Bhalerao 2007). However, by the end of October neither species showed increased stem freezing tolerance (Fig. 2), as indicated by LT_{50} values equivalent to values previously determined in non-hardened stems of the same two varieties grown at 20°C and an 18-h photoperiod (Pagter et al. 2008a). This suggests that the increased hardiness observed in late November was primarily because of decreasing temperature. Hence, in *Hydrangea* cold acclimation may not follow the common two- or three-stage cold acclimation process of other deciduous woody perennials (Gusta et al. 2005, Weiser 1970). In *Hydrangea*, SD may trigger growth cessation and dormancy induction, whereas increased freezing tolerance requires decreasing temperatures. SD alone has previously been shown to be ineffective in acquisition of cold hardiness in some plants such as rhizomes and buds of cloudberry and stems and buds of raspberry (Kaurin et al. 1982, Palonen 2006). Although both *H. macrophylla* and *H. paniculata* ceased growth and formed terminal buds which usually occur before and concurrent with dormancy development (Rohde and Bhalerao 2007), we did not provide evidence that they entered dormancy. However, as *Hydrangea* usually enters dormancy (Stuart 1958 in Smith and Kefford 1964), it is likely that termination of growth was the first SD-induced step toward establishing dormancy, which presumably potentiated LT-induced cold acclimation.

These results also suggest that cold acclimation in stems of both *H. macrophylla* and *H. paniculata* required temperatures below at least 5°C. Before October 25 the minimum air temperature reached was ca. 5°C, but between October 25 and November 25, when there was a significant increase in stem freezing tolerance in both species, several events below 5°C and two events of subzero temperatures occurred (Figs 1 and 2). A potential relationship between the occurrence of temperatures below ca. 5°C and cold acclimation was further strengthened by the correlations between number of hours with a mean temperature <5°C and LT_{50} values during acclimation (Table 1). These results are similar to those obtained by Adkins et al. (2003) for 10 different field-grown *H. macrophylla* varieties, where it was observed that in the beginning of November stem cold hardiness varied between −3 and −6°C. Approximately one month later, following two non-consecutive weeks of freezing temperatures, cold hardiness in all varieties had increased by at least −9°C. Hence, it is possible that the two episodes of subzero temperatures primarily acted as the triggering stimulus in cold acclimation of *Hydrangea* stems.

Interestingly, *H. macrophylla* and *H. paniculata* displayed very different trends in the xylem sap ABA concentrations during the seasons (Fig. 5). Cold acclimating conditions stimulated ABA biosynthesis in the roots of *H. paniculata*, and parallel increases in $[ABA]_{xylem}$ and stem freezing tolerance during acclimation suggests that ABA from the root system is involved in cold acclimation in *H. paniculata*. In *H. macrophylla* there was no direct positive relation between $[ABA]_{xylem}$ and stem freezing tolerance, making it doubtful whether ABA from the root system is functioning as a signal transducer in cold acclimating *H. macrophylla*. However, if $[ABA]_{xylem}$ increases transiently within days or few weeks, sampling at monthly intervals may be insufficient to observe any changes. Additionally, we cannot rule out that an environmental signal act via modulation of the sensitivity of cells to ABA rather than directly affecting the concentration of the hormone (Barros and Neill 1986). It is possible that increased $[ABA]_{xylem}$ are a direct effect of cold-induced dehydration, as low soil temperatures decrease root water absorption (Kramer and Boyer 1995) and desiccation of root tips results in increased $[ABA]_{xylem}$ (Loewenstein and Pallerdy 2002, Pagter et al. 2008b). However, insufficient root water uptake because of root chilling or soil freezing may primarily be a constraint in evergreen woody perennials. Additionally, because $[ABA]_{xylem}$ in *H. paniculata* decreased in late winter and early spring when the soil temperature was similar to the temperatures recorded in previous months, it seems unlikely that elevated $[ABA]_{xylem}$ were entirely a water-stress response induced by low soil temperature.

The stem water content decreased significantly in both species before any changes in freezing tolerance were observed (Fig. 3), suggesting that dehydration was not directly associated with cold acclimation. Instead decreased stem water content may have resulted from SD-induced dormancy development (Wake and Fennell 2000, Welling et al. 2002). The initial decrease in stem water content occurred at the same time as an increase in starch concentration (Fig. 6A), indicating that increasing

dry matter content accounted for at least part of the apparent water loss. Because reduced water content is commonly connected with increased hardiness (Guy 2003), it is possible that the observed differences in water content may partly explain the superior freezing tolerance of *H. paniculata* compared with *H. macrophylla* during deacclimation. Hence, in deacclimating *H. macrophylla* freezing tolerance was negatively associated with an increase in stem water content, whereas in *H. paniculata* an increase in stem water content lagged behind decreasing freezing tolerance.

In both *H. macrophylla* and *H. paniculata* the concentrations of sucrose and one of its galactosides, raffinose, were highly correlated with LT_{50} values indicating a putative role for these carbohydrates in freezing tolerance of *Hydrangea* (Fig. 6B and 6C, Table 1). Indeed, sucrose and raffinose have also been associated with freezing tolerance in other woody plants (Cox and Stushnoff 2001, Palonen et al. 2000, Renaut et al. 2004). The protective function of sucrose and raffinose may be attributed to their ability to stabilize membranes and proteins during freeze-induced dehydration (Crowe et al. 1998, Minorsky 2003). Accumulation patterns of glucose and fructose have frequently been shown to correlate with freezing tolerance in herbaceous plants (Gusta et al. 2004, Guy et al. 1992), whereas in woody plants the majority of studies have found no implication of hexoses in cold acclimation (Cox and Stushnoff 2001, Kasuga et al. 2007, Palonen et al. 2000, Sauter and Kloth 1987). Hence, it is noticeable that alterations in stem concentrations of fructose and glucose paralleled alterations in freezing tolerance in *H. macrophylla* but not in *H. paniculata* (Fig. 6D and 6E). Increasing concentrations of soluble carbohydrates and the development of cold hardiness coincided with decreasing concentrations of starch, indicating starch-to-sugar conversion (Fig. 6). Starch accumulated until leaf shedding, indicating that its breakdown was LT rather than SD induced, as previously suggested for other woody plants (Sauter and Kloth 1987, Sauter and van Cleve 1994). No relationship between genotype hardiness and concentrations of soluble carbohydrates was observed, as the least cold hardy species presented the highest concentrations of soluble carbohydrates. Hence, the nature of the accumulated soluble carbohydrates and/or the timing of accumulation may be more critical than the absolute concentrations. The observed differences in carbohydrate metabolism may be attributed to contrasting species-specific metabolic traits.

Distinct seasonal protein patterns were observed in bark of both *H. macrophylla* and *H. paniculata* (Fig. 4). As cold acclimation and dormancy development often occurs at the same time in woody perennials, it may be difficult to ensure that qualitative protein changes are specifically related only to increased freezing tolerance. However, as the first steps toward establishing dormancy (i.e. growth cessation) appeared weeks before a significant increase in stem freezing tolerance was observed, onset of cold acclimation and dormancy induction may have been somewhat separated in time in the present study. A clear correlation between increased freezing tolerance and accumulation of 22- and 27-kDa proteins in *H. paniculata* observed in November through March suggests that those proteins might be directly related to freezing tolerance. Additionally, expression of a ca. 34-kDa protein, which was nearly absent in September–October and in May, was also correlated with increased freezing tolerance in *H. paniculata*. Although speculative the autumnal increase in $[ABA]_{xylem}$ paralleled increases in freezing tolerance and expression of the 22-, 27- and 34-kDa proteins suggesting that they may be ABA inducible (Figs. 2, 4 and 5).

The most apparent seasonal pattern in proteins observed in both species, although more pronounced in *H. macrophylla* than in *H. paniculata*, was the upregulation of a 60-kDa protein during autumn followed by a subsequent downregulation during spring (Fig. 4). The identity of the 60-kDa protein is unknown, but dehydrins of the same size have been demonstrated in peach bark (Arora et al. 1996), blueberry floral buds (Muthalif and Rowland 1994), needles of Scots pine (Kontunen-Soppela et al. 2000) and leaves of poplar (Renaut et al. 2005). Dehydrins have been hypothesized to minimize deleterious dessicative effects associated with extracellular ice formation (Close 1996). In *H. macrophylla* an 18-kDa protein accumulated from November through February, when plants were most hardy, decreased sharply in March but returned to a higher level in May. This fluctuation was not considered as an irregularity as the pattern was observed repeatedly in separate extractions and SDS-PAGE analysis of replicate samples (data not shown). In 1-year-old shoots of peach, Wisniewski et al. (2004) also reported a sharp drop in the amount of a seasonally expressed 16-kDa storage protein in February followed by a subsequent increase in March. They noted that expression and turnover of storage proteins is a dynamic process, which can be influenced by several factors, potentially resulting in fluctuations in their induction and accumulation. Although the identity of the 18-kDa bark protein observed in *H. macrophylla* is unknown, its accumulation pattern fits the pattern described for bark storage proteins, being present in large quantities in winter and nearly or completely absent during summer (Wetzel et al. 1989). The current study represents a precursory examination of qualitative protein changes associated with cold acclimation in *H. macrophylla* and

H. paniculata. The importance of these changes and the identity and function of the observed proteins requires further studies.

When extracting *H. paniculata* samples the two protein extraction methods used were comparable in terms of 1-D gel resolution, whereas when used on *H. macrophylla* samples phenol-based extraction was superior to borate buffer extraction, indicating that bark tissue of *H. paniculata* contains less interfering compounds than those of *H. macrophylla*. Phenol extraction has previously been shown to have a high clean-up capacity. In addition to its selectivity as a solvent, phenol is a strong dissociating reagent decreasing molecular interactions between proteins and other materials (Faurobert et al. 2007). In phenol-based extraction two phases are formed, a lower aqueous phase containing carbohydrates, nucleic acids and insoluble cell debris, and an upper phenol rich phase containing cytosolic and membrane proteins, lipids and pigments (Carpentier et al. 2005). Considering the high concentrations of soluble carbohydrates in stems of *H. macrophylla* compared with stems of *H. paniculata* (Fig. 6B–E), we hypothesize that the species-specific difference in subsequent gel quality when using borate buffer extraction may be related to the observed differences in carbohydrate content of the two species, as the phenol-based method removes sugars from the proteins, whereas the borate method might not. The high sugar content in stems of *H. macrophylla* compared with *H. paniculata* was further evident when extracting bark proteins, as the initial extracts of *H. macrophylla* were extremely viscous and syrupy, whereas similar extracts of *H. paniculata* bark were much more fluid.

In summary, our results suggest that cold acclimation in *H. macrophylla* and *H. paniculata* is mainly regulated by LT specifically those below at least 5°C. Acclimation and deacclimation appeared to be approximately synchronized in the two species; however, they differed significantly in the level of mid-winter hardiness, rates of acclimation and deacclimation and physiological traits conferring tolerance to freezing conditions. In both *H. macrophylla* and *H. paniculata* cold acclimation was correlated with starch hydrolysis, accumulation of soluble carbohydrates and qualitative changes in bark proteins, but the two species displayed clear species-specific differences in seasonal accumulation patterns of specific soluble carbohydrates and protein profiles. In *H. paniculata* simultaneous increases in $[ABA]_{xylem}$, freezing tolerance and expression of certain proteins were observed, suggesting an involvement of ABA in induction of these processes. In contrast, ABA from the root system did not appear to be involved in cold acclimation in *H. macrophylla*, at least not in a concentration-dependent manner. Differences in freezing tolerance between the two *Hydrangea* species may therefore be related to differences in regulation of freezing tolerance.

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